SEX MAINTENANCE AND HORMONAL REGULATION OF ADULT SOMATIC STEM CELLS IN THE *DROSOPHILA* TESTIS AND OVARY

by

Qing Ma

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ABSTRACT

Local and systemic signals maintain adult stem cells in many tissues. Stem cells, in turn, form the basis of adult tissue regeneration by dividing asymmetrically to produce both stem cells and daughter cells that differentiate. The *Drosophila* gonads provide excellent model systems for studying adult stem cell behavior *in vivo*. In the testis, a single niche created by quiescent somatic hub cells maintains two types of stem cells: sperm-producing germline stem cells (GSCs) and supporting somatic stem cells called cyst stem cells (CySCs). Both types of stem cells attach to the hub, and divide asymmetrically to produce differentiating progeny that are displaced from the niche. The Drosophila ovary also contains a stem cell niche (created by quiescent somatic cap cells) which supports asymmetrically dividing GSCs. The ovarian somatic stem cells, called follicle stem cells (FSCs), produce follicle cells that envelop the differentiating germ cells and support germline differentiation. The differentiated somatic cells in the adult mammalian testis and ovary were recently shown to transdifferentiate to cells of opposite sex upon loss of sex-specific transcription factors. Whether the sexual identity of adult stem cells in the Drosophila gonads is also maintained was not known. Also, little was known about the roles of hormonal signaling in Drosophila testis stem cell function.

In the *Drosophila* testis niche, local cytokine signaling promotes somatic cyst stem cell (CySC) renewal through several effectors, including the putative transcription factor Chronologically inappropriate morphogenesis (Chinmo). I found a new function for Chinmo in preventing the feminization of adult CySCs. When I reduced Chinmo levels in all of the adult CySCs, the testis somatic cells underwent a progressive transformation into cells similar to ovarian somatic cells. In the earliest stages of sex conversion, CySCs lost expression of the

canonical male sex determination factor DoublesexM (Dsx^M) and gained expression of the FSC marker Castor. Later, the testes with reduction of Chinmo developed a layer of columnar epithelium similar to the follicle cell layer in the ovary; these cells, called follicle-like cells, expressed various follicle cell markers. In addition, ectopic expression of Dsx^M in the CySC lineage partially rescued the *chinmo* sex transformation phenotype, and knocking down doublesex (*dsx)* in the CySCs partially phenocopied *chinmo*, placing Chinmo upstream of Dsx^M. In the ovary, ectopic *chinmo* expression was sufficient to promote male somatic identity through a Dsx^M-independent mechanism. Similarly, overexpressing female sex determination factors caused early but not later aspects of the *chinmo* phenotype to develop, which suggested that the female sex determination factors may feminize male somatic cells in parallel with Chinmo. I also showed that ectopic expression of the miRNA *let-7*, which targets Chinmo in the Drosophila brain, downregulates Chinmo in testes strongly enough to feminize the testis, but *let-7* is not required in the ovary. My work is consistent with the finding that the mammalian Dsx homologue Doublesex and mab3-related transcription factor 1 (DMRT1) prevents the male-to female conversion of differentiated somatic cells in the adult mammalian testis. It is also consistent with the finding that DMRT1 is also sufficient to masculinize the female somatic cells in the gonad.

To study the effect of systemic signals on stem cells in the Drosophila testis, we examined the Drosophila ecdysone pathway, which is a well-characterized steroid-hormone signaling pathway that coordinates embryogenesis, larval molting, and metamorphosis. We found that the ecdysone hormone and ecdysone signaling pathway members are required for CySC maintenance, and that GSCs non-autonomously require ecdysone signaling.

My work showed that sex maintenance occurs in adult somatic stem cells in both the testis and the ovary in Drosophila and contributes to the understanding of how local niche signals and systemic signals regulate stem cell fate.

Thesis advisor: Dr. Erika Matunis

Reader: Dr. Geraldine Seydoux

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CHAPTER 1

General Introduction

Stem cell niches in the *Drosophila* testis and ovary

Stem cells have the unique ability to divide asymmetrically to produce one self-renewing daughter that remains a stem cell and one daughter that will give rise to a more differentiated type of cell. Adult stem cells play an important role in tissue maintenance, regeneration and repair. Stem cell populations typically reside in niches, which are specialized microenvironments that regulate how stem cells function. Adult stem cell niches are present in multiple tissues including blood, brain, skin and testis. But mammalian niches and stem cells are hard to identify because of the complexity of the tissues and lack of stem cell-specific markers. Also the molecular regulation of the stem cell niches is not very clear. In Drosophila, the ovary and testis stem cell niches are very well defined and studied systems. The high conservation between the mammalian stem cell niches and Drosophila gonad stem cell niches allows us to study stem cell niches using the Drosophila gonads as a model system.

In the *Drosophila* testis, a group of non-mitotic cells called the hub (Figure 1.1A yellow) functions as an important signaling center to maintain the surrounding male somatic stem cells called cyst stem cells (CySCs, Figure 1.1A, dark blue cells) and male germline stem cells (GSCs, Figure 1.1A dark green cells) (Tulina 2001, Leatherman and DiNardo 2010) (Figure 1.1). GSCs make broad contact with the hub and divide asymmetrically to give rise to gonialblast daughter cells. Gonialblasts divide four times to generate clusters of spermatogonia, which will further differentiate to become sperm. Approximately two CySCs surround each GSC and make small contacts with the hub. CySCs divide asymmetically to generate cyst cells. A pair of cyst cells forms a septate junction to envelop each gonialblast. Cyst cells exit the cell cycle but grow to continually encyst the gonialblast and its descendants as they differentiate.

The ovarian germline stem cell niche has cap cells supporting the self-renewal of 2-3 GSCs (Figure 1.1B, dark pink cells), which produce differentiating female germ cells (Figure 1.1B, light pink cells). Escort cells surround the GSCs. Two follicle stem cells (FSCs, Figure 1.1B, red cells) are positioned posteriorly and opposite one another near the middle of the germarium, producing follicle precursor cells (magenta). Follicle precursor cells differentiate into somatic follicle cells (Figure 1.1B, orange cells), stalk cells (purple) and polar cells (yellow). Follicle cells form a columnar epithelium to envelop each 16-cell cyst and the germline cyst along with its associated somatic cells forms an egg chamber. Egg chambers are linked by chains of stalk cells, and polar cells are located at the anterior and posterior ends of the egg chamber. The morphology and behavior of somatic cells in the ovary and testis are quite distinct: the male CySCs produce squamous progeny called cyst cells, which are quiescent, while female FSCs produce columnar epithelial cells which can proliferate.

Jak/STAT signaling and Chinmo in the testis

The highly conserved Janus kinase-Signal Transducer and Activator of Transcription (Jak-STAT) pathway regulates stem cell maintenance in the *Drosophila* testis. The hub secretes the Jak-STAT ligand Unpaired (Upd); Upd binds to the receptor Domeless on GSCs and CySCs, leading to the activation of Jak kinase and its target STAT in both types of stem cell (Figure 1.2). STAT, a transcription factor, activates target genes leading to maintenance of GSCs and CySCs (Tulina 2001, Leatherman and DiNardo 2010). Several Jak-STAT targets in CySCs, such as Zinc-finger homeodomain-1 (Zfh-1) and Chronologically inappropriate morphogenesis (Chinmo), are required for CySC self-renewal (Leatherman and Dinardo 2008, Flaherty, Salis et al. 2010, Issigonis and Matunis 2012, Michel, Kupinski et al. 2012, Amoyel,

Sanny et al. 2013). Chinmo is a BTB-zinc figure domain protein required for CySC (but not GSC) self-renewal (Flaherty, Salis et al. 2010). It was first identified in the *Drosophila* brain and is required for the correct timing of neuroblast differentiation and for neuronal temporal identity (Zhu, Lin et al. 2006, Flaherty, Salis et al. 2010). Here we found an unexpected function of Chinmo in sex maintenance of CySCs: it promotes the male identity of adult CySCs through the canonical male sex determination pathway.

Somatic sex determination and sex maintenance

Various genetic and environmental signals control sex determination and sexual differentiation, which result in sex dimorphism in the morphology, physiology and behavior of animals. These sex determination signals include sex-specific chromosomes (XX versus XY), temperature, social cues, and the haplodiploid system (for example, in bees) (Matson and Zarkower 2012). The molecular mechanism of sex determination also varies across taxa: alternative splicing, translational regulation, and extracellular signaling are some examples (Matson and Zarkower 2012). But in a lot of species, the downstream regulation converges on genes that contain a conserved DM domain, such as *doublesex (dsx)* in *Drosophila*, *male abnormal 3* (*mab-3)* in *C. elegans,* and Dsx- and mab-3-related transcription factor 1 *(Dmrt1*) in vertebrates (Figure 1.3). In mammals and most vertebrates, genes involved in sex determination like *Dmrt1* are first expressed in the somatic cells of the embryonic gonad and this leads to the primary sex determination of the gonad (Figure 1.3). Then, hormones produced by the gonads direct the sex determination and differentiation of the rest of the body. Sexual phenotypes were thought to be determined exclusively by systemic hormones, but recent studies have found exceptions to this rule. In birds, for example, somatic sexual identity

may be largely cell autonomous, as illustrated by lateral gynandromorph chickens; these male:female chimeras have one side that appears male and the other female, even though both sides are exposed to the same blood system and hormonal environment (Zhao, McBride et al. 2010). Also, the sexual identities were long thought to be set after initial sex determination. In mammals, the choice was found to be labile even in adult gonads and is controlled by intrinsic factors; loss of sex-specific transcriptional regulators in the adult mouse gonad causes differentiated somatic cells to transdifferentiate into somatic cells of the opposite sex (Uhlenhaut, Jakob et al. 2009, Matson, Murphy et al. 2011). This indicates that sexual identity must continuously be maintained in specific differentiated cell types long after sex determination has occurred. Our recent work in Drosophila showed that the sexual identity of adult somatic stem cells in the testis also has to be actively maintained (Ma, Wawersik et al. 2014). Since adult stem cells have the capacity to rebuild entire adult organ systems, altering a stem cell's sexual identity could conceivably cause widespread changes to the tissue.

In *Drosophila*, a well-characterized alternative-splicing cascade that acts through the splicing factor Sex lethal (SXL) is required for both germline and somatic sex determination, but acts through different targets in each lineage (Figure 1.4). The *transformer* (*tra*) and *dsx* genes control somatic sex determination and PHD finger protein 7 (Phf7) is thought to be one of the SXL targets in the germline (Yang, Baxter et al. 2012). In addition, the Jak-STAT pathway is required for the initial sex determination of male somatic cells in the testis, and the somatic cells promote the developing germ cells to acquire a male identity later (Jinks 2000, Wawersik, Milutinovich et al. 2005). But it was not known whether sexual identity has to be actively maintained in adult *Drosophila* (as has recently been found in mammals). And the requirement for the Jak-STAT pathway in somatic sex maintenance was not known. Here we

found that maintenance of male somatic identity is required in somatic stem cells within the adult *Drosophila* testis, and this process requires the Jak-STAT pathway member *chinmo*. Also we found that Chinmo is sufficient to promote the male identity in follicle stem cells in the adult ovary. In Chapter 2, I will describe the function of Chinmo in maintaining male identity of stem cells in adult testes. In Chapter 3, I will describe the role of Chinmo in adult ovaries and the function of the canonical sex determination pathway in sex maintenance in both testes and ovaries.

Hormonal regulation of stem cells

Local signals are known to regulate stem cells; however, the roles of systemic signals in stem cell function are largely uncharacterized. In mammalian testes, follicle-stimulating hormone promotes expression of the stem cell maintenance factor GDNF to regulate SSC proliferation (Tadokoro, Yomogida et al. 2002). Other recent work has found that hematopoietic stem cells, which were thought to function similarly in both sexes, respond to the higher levels of estrogen in female mice, resulting in differences in the self-renewal ability of hematopoietic stem cells in male and female mice (Nakada, Oguro et al. 2014). But how systemic and local signals cooperate to regulate stem cells is not clear and it is difficult to investigate in complex mammalian systems. In *Drosophila*, hormonal signals such as insulin, juvenile hormone, and ecdysone are important throughout development and in adults (Ables, Laws et al. 2012). The steroid hormone twenty-hydroxyecdysone (20E), which is an active form of ecdysone hormone, is required for coordinating development including embryogenesis, larval molting, puparium formation, and metamorphosis (Baehrecke 1996, Yamanaka, Rewitz et al. 2013). 20E binds to *Ecdysone receptor* (*EcR*), which is the

orthologue of the mammalian *farnesoid X receptor*/*liver X receptor* and forms a heterodimeric nuclear hormone receptor complex with *ultraspiracle* (*usp*), which is the orthologue of the mammalian *retinoid X receptor* (Hayward, Bastiani et al. 1999, King-Jones and Thummel 2005). These nuclear hormone receptors have ligand binding domains (LBDs) and DNA binding domains (DBDs), which can bind to hundreds of target genes and activate or repress them in response to cell-type-specific co-activators (Tsai, Kao et al. 1999, Perera, Zheng et al. 2005, Jang, Chang et al. 2009, Francis, Zorzano et al. 2010, Carbonell, Mazo et al. 2013) (Figure 1.5). Although ecdysone signaling has been studied primarily during development, 20E is also present in adult *Drosophila* at very low levels (Hodgetts, Sage et al. 1977, Handler 1982, Bownes, Dubendorfer et al. 1984, Kozlova and Thummel 2000). This low level of 20E in adult flies has been shown to play roles in sleep, longevity and female reproduction (Carney and Bender 2000, Ishimoto, Sakai et al. 2009, Tricoire, Battisti et al. 2009, Ishimoto and Kitamoto 2010). Ecdysone is also required female germline stem cell maintenance in adult flies (Ables and Drummond-Barbosa 2010). Although adult female flies contain detectable levels of ecdysone there is much less of this hormone present in adult males. But here we show that the ecdysone pathway is expressed, activated and also required in the adult testis for CySC maintenance as I will describe in Chapter 4.

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Figure Legends

Figure 1.1 Illustration of a wild-type *Drosophila* testis and a wild-type *Drosophila* ovary

(**A**) Illustration of a wild-type *Drosophila* testis (right) with the apex magnified (left). Germline stem cells (GSCs, dark green) and somatic cyst stem cells (CySCs, dark blue) adhere to the hub (yellow). GSCs produce differentiating male germ cells (spermatogonia and spermatocytes, green) that are displaced from the hub and form elongated spermatids (grey) and mature sperm (not shown). Approximately two CySCs flank each GSC; CySCs produce squamous, quiescent cyst cells (light blue), which encase differentiating germ cells. **(B)** Illustration of a wild-type *Drosophila* ovariole (top) comprised of a germarium (magnified, bottom) followed by a series of developing egg chambers. In the germarium, anterior niche cells (cap cells, grey) support GSCs (dark pink), which produce differentiating female germ cells (light pink). Two somatic follicle stem cells (red), located near the middle of the germarium, produce follicle precursor cells (magenta), which differentiate into follicle cells (orange), stalk cells (purple), and polar cells (yellow). Each egg chamber contains 16 germ cells surrounded by a monolayer of columnar epithelial follicle cells. Polar cells are located at each end; egg chambers are linked by chains of stalk cells.

Figure 1.2 Chinmo is a Jak/STAT effector required for CySC self-renewal in the testis

The hub secretes the Jak/STAT ligand Unpaired (Upd); Upd binds to the receptor on GSCs and CySCs, leading to the activation of Jak kinase and its target STAT in CySCs (and GSCs, not shown). STAT activates target genes like Chinmo leading to CySC self-renewal. Chinmo is a BTB-zinc finger domain protein. mZPF509 is a mammalian homologue for Chinmo.

Figure 1.3 Vertebrate sex determination and the roles of DMRT1 orthologues

Blue shading indicates masculinizing function and pink indicates feminizing function. In mammals, the sex-determining region of chromosome Y (SRY) initiates male development. DMRT1 is expressed in the gonadal somatic cells but it's not required for the initial sex determination. It is required only after gonadal sex is determined. In other vertebrates like fish, chicken and frog, DMRT1 and its orthologues are required for both male sex determination and differentiation.

Figure 1.4 Somatic sex determination in Drosophila

An alternative splicing cascade controlled by the upstream regulator *sxl* leads to alternative splicing of *tra* and *dsx*. The female form of Dsx (Dsx^F) promotes female identity and the male form of Dsx (Dsx^M) promotes male identity of somatic cells.

Figure 1.5 Diagram of the Drosophila ecdysone pathway

The hormone twenty-hydroxyecdysone (20E, blue dots) activates ecdysone signaling by binding to a heterodimer composed of EcR and USP. EcR and USP each contain a ligand binding domain (LBD) and a DNA binding domain (DBD). When 20E binds EcR and forms a complex with USP, the complex then recognizes ecdysone response elements (EcREs) within the genome to regulate downstream gene expression (pink dots).

Figure 1.1

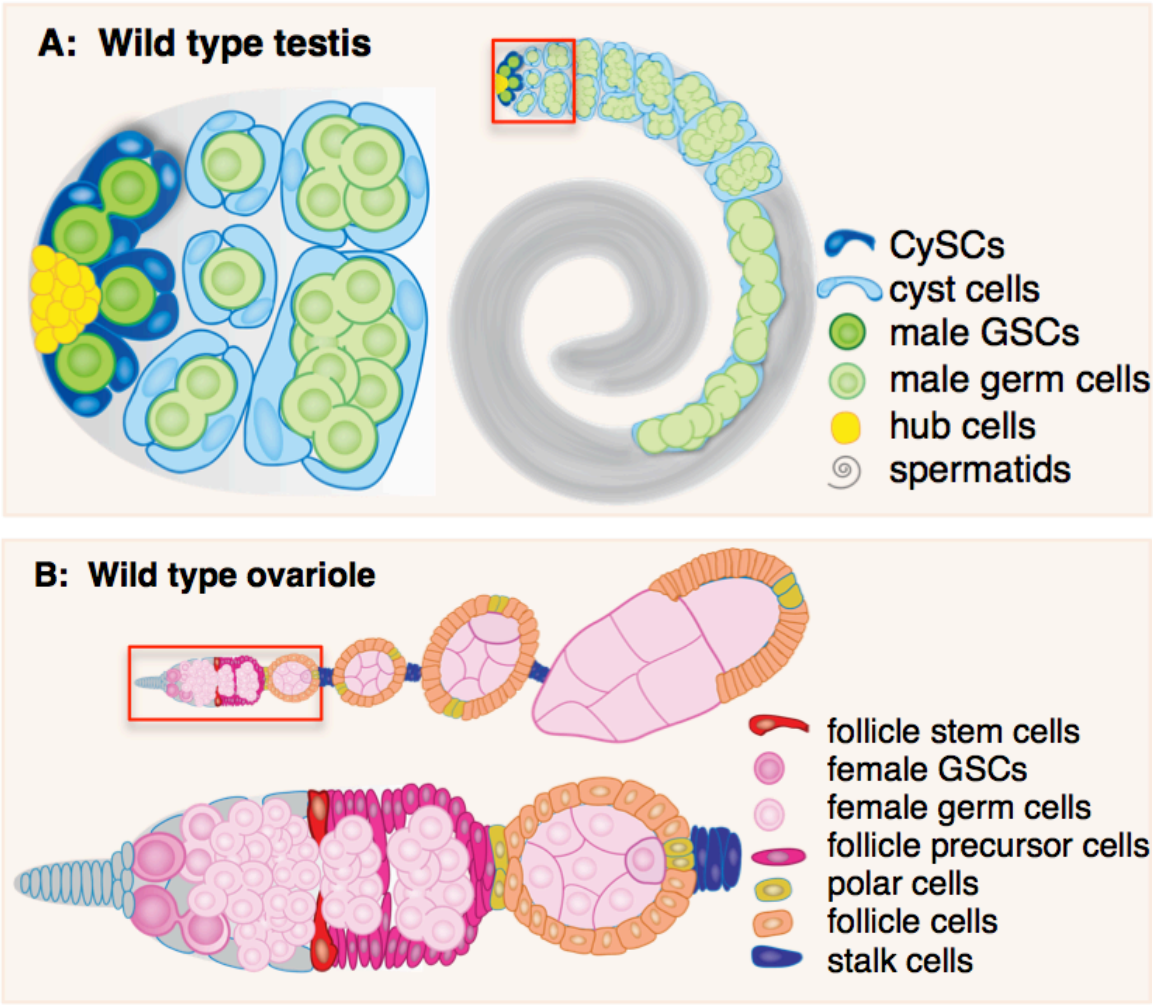

A: Wild type testis
CySCs
cyst cells
male GSCs
male germ cells
hub cells
spermatids
B: Wild type ovariole
follicle stem cells
female GSCs
female germ cells
follicle precursor cells
polar cells
follicle cells
stalk cells

Figure 1.2

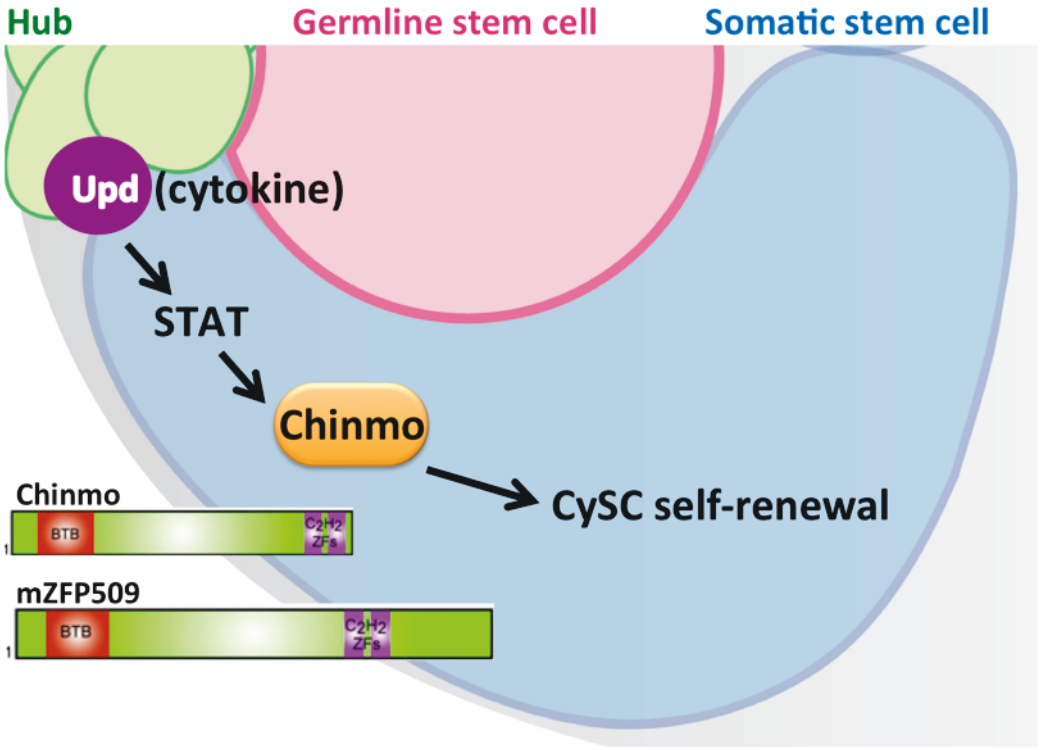

Hub
Germline stem cell
Somatic stem cell
Upd (cytokine)
STAT
Chinmo
CySC self-renewal
Chinmo
BTB
C2H2 ZFs
mZFP509
BTB
C2H2 ZFs

Figure 1.3 (From Matson and Zarkower, 2012)

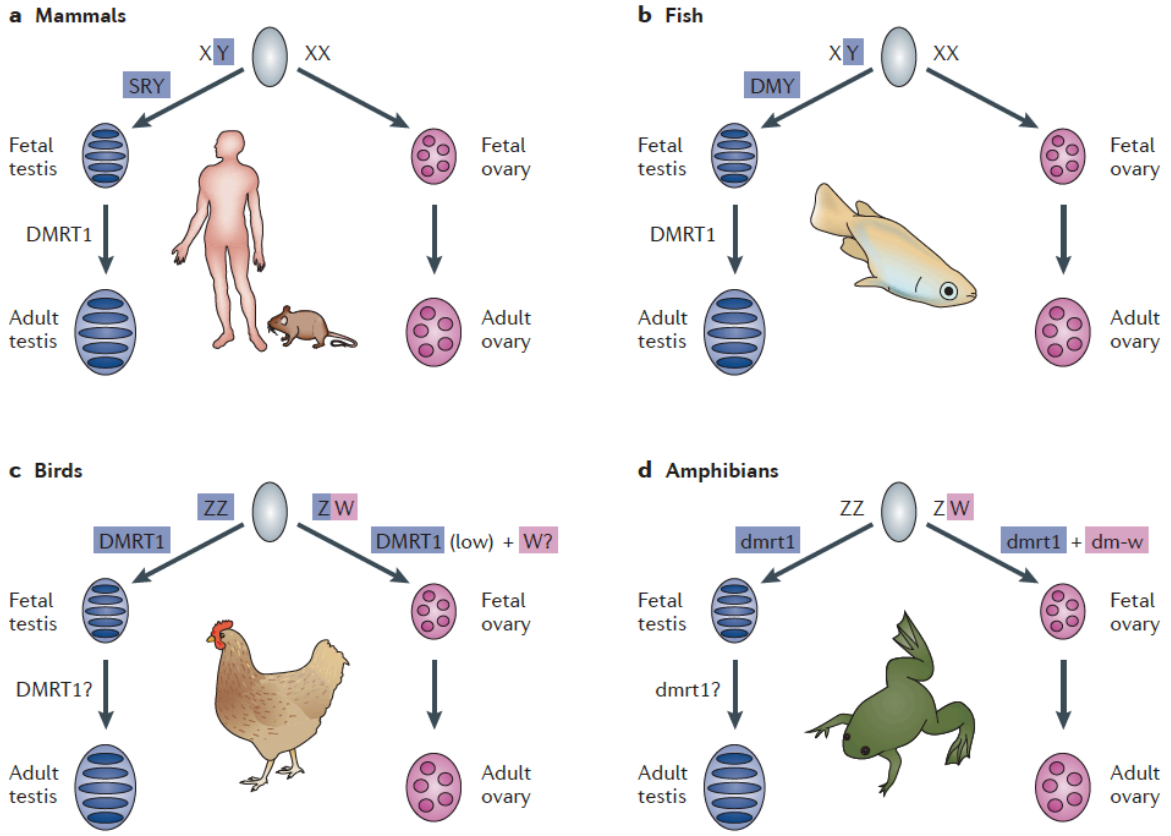


Figure 1.4

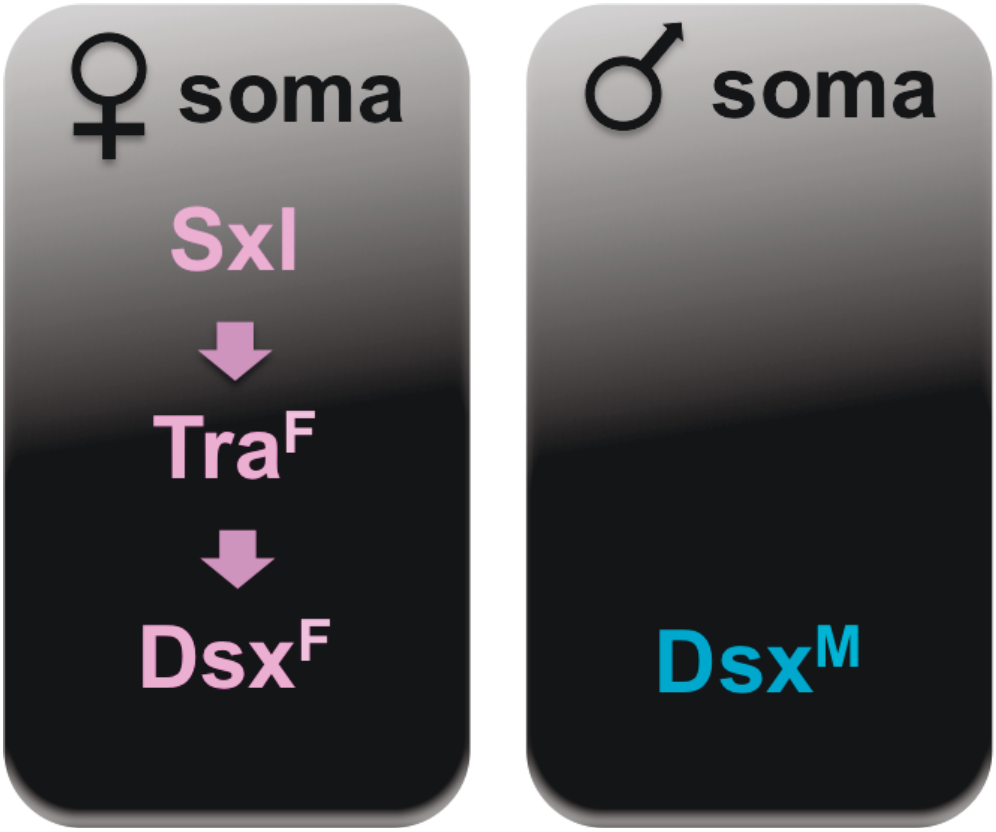
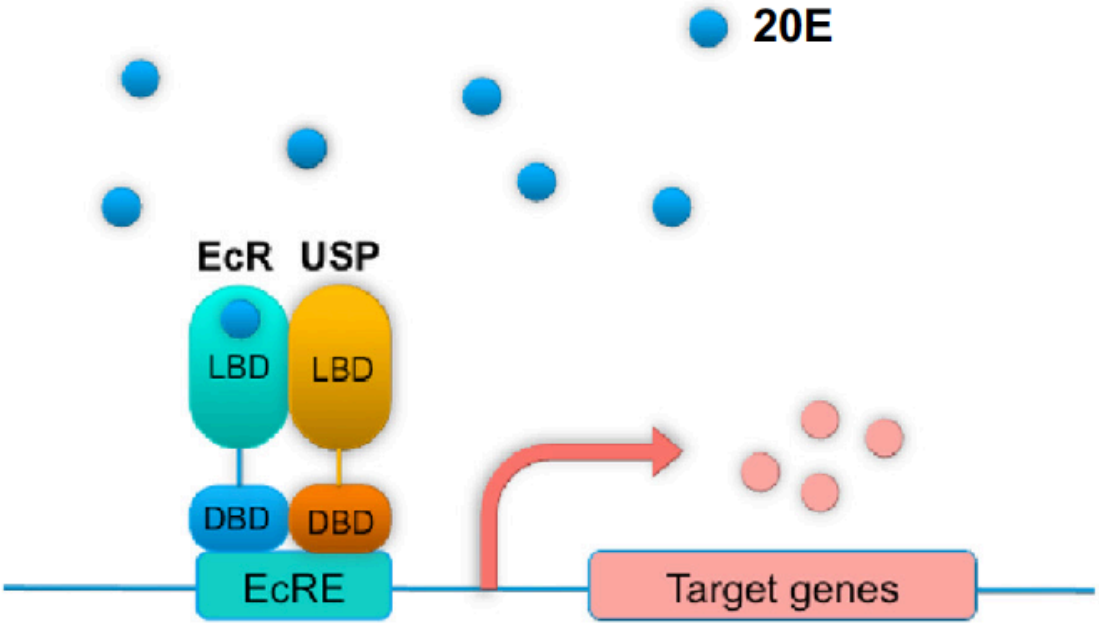


Figure 1.5



Chapter 2

The Jak-STAT target Chinmo prevents sex transformation of adult stem cells in the Drosophila testis niche

This chapter is a modified version of the manuscript, "Ma Q, Wawersik M, Matunis E. (2014) The Jak-STAT target Chinmo prevents sex transformation of adult stem cells in the Drosophila testis niche. Developmental Cell 31: 474-486"

Summary

Adult stem cell populations in many tissues, are regulated by signaling from the stem cell local microenvironment, or niche, but it was not known whether sexual identity of adult stem cells must also be maintained. In the *Drosophila* testis niche, local activation of the Janus kinase-Signal Transducer and Activator of Transcription (Jak-STAT) pathway maintains two types of stem cells: sperm-producing germline stem cells (GSCs) and supporting somatic stem cells called cyst stem cells (CySCs). Here, we show that the Jak-STAT effector *chronologically inappropriate morphogenesis* (*chinmo*), which encodes a transcription factor required for CySC maintenance, also prevents feminization of somatic stem cells in the adult *Drosophila* testis. Chinmo promotes expression of the canonical male sex determinant DoublesexM (Dsx^M) within CySCs and their progeny, and ectopic expression of DsxM in the CySC lineage partially rescues the *chinmo* sex transformation phenotype, placing Chinmo upstream of Dsx^M. The Dsx homologue DMRT1 prevents the male-to-female conversion of differentiated somatic cells in the adult mammalian testis, but its regulation is not well understood. Our work indicates that sex maintenance occurs in adult somatic stem cells, and that this highly conserved process is governed by effectors of stem cell niche signals. Analysis of Chinmo and Dsx/Dmrt1-mediated sex maintenance pathways in somatic stem cells may also give insight into maintenance of sexual dimorphism in other organs, and provide a unique model to study adult stem cell transdifferentiation in vivo

Introduction

Male versus female fate is controlled by a variety of mechanisms across taxa (Kopp 2012). In mammals, this choice was recently found to be labile even in adults; loss of sex-specific transcriptional regulators in the adult mouse gonad causes differentiated somatic cells to transdifferentiate into somatic cells of the opposite sex (Uhlenhaut, Jakob et al. 2009, Matson, Murphy et al. 2011). This indicates that sexual identity must continuously be maintained in specific differentiated cell types long after sex determination has occurred. Whether sexual identity is plastic in undifferentiated adult stem cells remains unknown. Since adult stem cells have the capacity to rebuild entire adult organ systems, altering a stem cell's sexual identity could conceivably cause widespread changes to the tissue.

In *Drosophila*, a well-studied sex determination cascade culminates in the sex-specific splicing of mRNA encoding the conserved transcription factor Doublesex (Dsx); this binary choice cell-autonomously dictates somatic sexual identity (Whitworth, Jimenez et al. 2012). While upstream regulation of sex determination differs between flies and mammals, downstream control converges on *Dsx/mab-3 related transcription factor* (*Dmrt*) genes (Matson and Zarkower 2012). The Janus kinase-Signal Transducer and Activator of Transcription (Jak-STAT) pathway is also required for the initial choice of somatic sexual identity in *Drosophila* embryos and promotes male germline sexual behavior in embryonic testes (Jinks, Polydorides et al. 2000, Wawersik, Milutinovich et al. 2005). However, it is not known whether Jak-STAT signaling is required for sex maintenance in *Drosophila,* and the link between the Jak-STAT pathway and the canonical sex determination pathway is unknown.

The *Drosophila* ovary and testis provide excellent models for studying adult stem cell behavior *in vivo* (Fuller and Spradling 2007, Matunis, Stine et al. 2012). In the testis, Jak-STAT signaling maintains two types of stem cells: sperm-producing germline stem cells (GSCs) and supporting somatic stem cells called cyst stem cells (CySCs). Both of these cell types attach to a single niche created by quiescent somatic hub cells at the testis apex and divide asymmetrically to produce differentiating progeny (spermatogonia and cyst cells, respectively) that are displaced from the niche (Matunis, Stine et al. 2012). Several factors, including the Jak-STAT targets Zinc-finger homeodomain-1 (Zfh-1) and Chinmo, are required for CySC self-renewal (Leatherman and Dinardo 2008, Flaherty, Salis et al. 2010, Issigonis and Matunis 2012, Michel, Kupinski et al. 2012, Amoyel, Sanny et al. 2013). Here we reveal an unexpected function of Chinmo: it acts through the canonical sex determinant Dsx^{M} to maintain the male identity of adult CySCs.

Results

Reduction of Chinmo triggers the appearance of cells resembling ovarian follicle cells in the adult niche, then throughout the testis

While screening for *Drosophila* testis phenotypes, we identified a spontaneous mutation causing a striking transformation of the adult testis. Adult mutant males are fertile, indicating testes develop normally. Consistent with this observation, testes from young males (0-1 day) are indistinguishable from wild type testes in overall morphology (Figures 2.1C-D, I-J). With age, however, a progressive change in the testis morphology occurs. Initially, subtle changes are detected at the testis apex, where aggregates of epithelial somatic cells (defined as 8 or more closely apposed cells expressing high levels of adhesion proteins) appear adjacent to the hub, while the remainder of the tissue is unaffected (Figures 2.1E, K, P-Q). With time, somatic cell aggregates acquire additional cells and extend away from the testis apex, while older differentiating germ cells and cyst cells are displaced toward the basal end of the testis (Figures 2.1F-G, L-M). In 7-9 day old males, an obvious transformation is apparent throughout the testis: somatic cell aggregates adjacent to the hub remain but now a monolayer of columnar epithelial cells lines the testis periphery, while germ cells are restricted to the lumen of the tissue (Figures 2.1G, M, R). The progression of this phenotype from the testis apex to the basal end suggests a stem cell origin. This testis phenotype had not been described before. However, the somatic cells bear a striking resemblance to the arrangement of somatic follicle cells within the *Drosophila* ovary, which form a columnar monolayer surrounding developing germ cells (Mahowald and Kambysellis 1980) (Figures 2.1B, H, N, S). Therefore, we refer to these somatic cells in the mutant testes as “follicle-like cells”. We also find that germ cells in 7-9 day old mutant testes are arrested as early male germ cells (spermatogonia)

based on their morphology, branching fusomes (Lin and Spradling 1994, Hime, Brill et al. 1996, de Cuevas, Lilly et al. 1997) (open arrowheads, Figure 1R), expression of the male-specific early germ cell marker M5-4 (Tran, Brenner et al. 2000) (Figures S2.1A-C) and the undifferentiated germ cell marker Bam (data not shown) (McKearin and Spradling 1990). By 2-3 weeks, degeneration of differentiation-arrested germ cells is observed (Figure S2.1D). Since germ cell arrest occurs when the germline and somatic sex are mismatched (Whitworth, Jimenez et al. 2012), this phenotype is consistent with a defect in somatic sexual identity maintenance in the adult testis.

Sex maintenance has not been characterized previously in the adult *Drosophila* testis or ovary, but was recently found to occur in the adult mammalian testis and ovary (Uhlenhaut, Jakob et al. 2009, Matson, Murphy et al. 2011). The underlying mechanisms are of interest, since defects in this process may cause testicular cancer or disorders of sexual development (Hanson and Ambaye 2011). Therefore, we sought to identify this spontaneous mutant. Our genetic complementation (Table S2.1, Figure 2.2A) and rescue experiments (Figures. 2.2B-C, Table S2.2) indicate that the mutation is a partial loss-of-function allele of the essential BTB-Zinc finger cell fate determinant *chinmo* (Zhu, Lin et al. 2006, Flaherty, Salis et al. 2010), which we denote $chinmo^{Sex\ Transformation}$ *, or* $chinmo^{ST}$. Furthermore, Chinmo is specifically depleted in the CySC lineage in $chinmo^{ST}$ mutant testes (Figures 2.2D-H, S2.1E-F). Together, these data support the hypothesis that Chinmo promotes male sexual identity in the adult testis soma.

Reduction of Chinmo in adult testes causes somatic stem cells and their progeny to acquire female somatic identity

Since testes with reduced *chinmo* acquire somatic cells that morphologically resemble ovarian follicle cells, we directly tested whether these follicle-like cells underwent a male-to-female sexual transformation. Specifically, female-specific gene expression and cell behavior was examined in $chinmo^{ST}$ testes. Castor, Cut, and a *Slbo-GFP* gene reporter are normally expressed in adult ovaries (Jackson and Blochlinger 1997, Chang, Jang et al. 2013) (described below), but not testes (Figures 2.3A, D, G). Castor, the earliest marker in the ovarian follicle stem cell lineage, marks follicle stem cells and their earliest progeny (Figure 2.3C), while more differentiated follicle cells express high levels of Cut and then *Slbo-GFP* (Figures 2.3F, I). In $chinmo^{ST}$ testes, these three markers are sequentially expressed as the phenotype progresses (Figures 2.3B, E, H). In testes from young (1-3 day) $chinmo^{ST}$ males, follicle-like cells have not formed yet (described above). However, the earliest ovarian marker, Castor, is detected in most testes (61.7%, n=47). Importantly, Castor is always restricted to a subset of somatic cells within the testis at this time-point: CySCs and their earliest daughters (Figure 2.3B). To ask whether Castor expression originates in CySCs, their earliest daughters, or both, we next stained testes from even younger males (newly eclosed virgins). At this time-point, fewer testes contained Castor-positive cells (38.8% of testes, n=49), and the number of Castor-positive cells ranged from 3 to around 30 (data not shown). Castor was detected in at least one CySC in all testes where it was expressed, further supporting the hypothesis that CySCs, but not their differentiating progeny (cyst cells) which have exited the stem cell niche, are the cells within the testis that undergo sexual transformation. These data further suggest that follicle-like cells arise from sex transformed CySCs. Consistent with this hypothesis, the ovarian somatic cell marker Cut becomes strongly expressed in follicle-like cells in 7-9 day old $chinmo^{ST}$ testes (Figure 2.3E). In contrast, the earlier marker, Castor, becomes depleted

from follicle-like cells (described below, Figure 2.4), a pattern consistent with its absence in ovarian follicle cells (Chang, Jang et al. 2013). Finally, the *Slbo-GFP* reporter, which normally becomes highly expressed in follicle cells at stage 10 of oogenesis (Cai, Chen et al. 2014) (Figure 2.3I), is also expressed in a subset of follicle-like cells in older $chinmo^{ST}$ testes (Figure 2.3H). Together these data suggest that follicle-like cells can progress through the normal follicle cell differentiation program to a surprising extent, in an otherwise male gonad. Consistent with this hypothesis, *Yolk protein1* (*Yp1*) transcripts, which become highly expressed in follicle cells at stage 10 but are not transcribed in the testis (Brennan, Werner et al. 1982, Logan, Garabedian et al. 1989), are present in testes with reduced *chinmo* (Figure 2.3J).

In addition to distinct morphological and molecular characteristics, somatic cells in the testis and ovary differ in potency and proliferation status. CySCs are unipotent, giving rise to cyst cells that exit the cell cycle (Gönczy and Dinardo 1996). In contrast, follicle stem cells are multipotent, yielding both stalk cells and mitotically active follicle cells (Margolis and Spradling 1995). By examining markers of cell proliferation in $chinmo^{ST}$ testes, we find that follicle-like cells express mitotic markers (Figures 2.3K-O). Furthermore, somatic cells with the molecular and morphological characteristics of stalk cells become apparent in older $chinmo^{ST}$ testes (Figure 2.4), suggesting that reduction of Chinmo causes CySCs to transdifferentiate into cells resembling multipotent female follicle stem cells. Together, these data support the hypothesis that *chinmo* actively maintains a male sexual identity in adult CySCs.

Chinmo autonomously maintains male sexual identity in adult somatic stem cells

The above data indicate that CySCs specifically require Chinmo for maintenance of male sexual identity. However, this requirement could be direct or indirect. To distinguish between these possibilities, we used cell-type specific RNA-interference (RNAi) knockdown of *chinmo*. Consistent with previous mosaic analysis (Flaherty, Salis et al. 2010), knockdown of *chinmo* in the germ line during development does not yield a testis phenotype (Figure 2.5F), ruling out a germline requirement for *chinmo*. We next allowed testes to develop normally, and then conditionally induced *chinmo* RNAi in subsets of adult somatic cells. Knockdown of *chinmo* in adult hub cells does not yield testes with the *chinmo*ST phenotype (Figure 2.5G). In contrast, the *chinmo*ST phenotype is fully recapitulated by knockdown of *chinmo* in all adult CySCs and early cyst cells (Figures 2.5A-E, S2.2A-D, Table S2.3a and b). To determine whether *chinmo* is required primarily in CySCs or in their cyst cell progeny, we compared the phenotypes that developed when various CySC lineage drivers were used to induce *chinmo* RNAi. Drivers expressed in CySCs and their immediate cyst cell daughters are sufficient to recapitulate the *chinmo*ST phenotype, while drivers expressed solely in later cyst cells are not (Figures 2.5H, S2.2E-I, Table S2.3a). Together these data indicate that *chinmo* is required in CySCs, but not in their differentiated progeny, for the maintenance of adult somatic sexual identity.

Follicle-like cells arise from the adult somatic stem cell lineage, not hub cells

The fact that CySCs autonomously require *chinmo* for maintenance of adult sexual identity suggests that follicle-like cells arise directly from CySC lineage cells. However, it is also possible that *chinmo* is required in CySCs to prevent hub cells, the only other somatic

cells in the testis niche, from giving rise to follicle-like cells. To distinguish between these possibilities, we used genetic lineage tracing to permanently mark hub cells or CySCs and early cyst cells in *chinmo*ST males during development, and then assayed for the presence of permanently marked cells in adult testes. Permanently marked hub cells do not produce marked follicle-like cells in *chinmo*ST testes (Figures 2.6A-B, Table S2.4), ruling out hub cells as a source of follicle-like cells. In contrast, testes with permanently marked CySCs and early cyst cells acquire marked follicle-like cells over time (Figures 2.6C-E). Since follicle-like cells do not express the CySC and early cyst cell driver used in this experiment (Figures S2.2J-L), the marked follicle-like cells must be descendants of CySCs and early cyst cells. We conclude that CySCs and early cyst cells, but not hub cells, give rise to follicle-like cells when *chinmo* is reduced. Consistent with this finding, hub cells within *chinmo*ST testes remained quiescent when assayed for proliferation using extended in-vivo BrdU labeling (Figures 2.6F-H). Since all CySC drivers are also expressed in early cyst cells, we cannot exclusively mark CySCs via lineage tracing. However, since CySCs but not cyst cells require *chinmo*, and CySCs are the earliest cells to express ovarian somatic markers, our data strongly support the hypothesis that somatic stem cells, rather than differentiated cells, undergo sex transformation when levels of Chinmo are reduced.

Chinmo maintains male sexual identity through the canonical sex determination pathway

Sex determination in both mammals and *Drosophila* converges on the transcription factor Dsx/Dmrt1. Our finding that *chinmo* promotes adult somatic sex maintenance prompted us to ask whether Chinmo works in concert with the canonical sex determination pathway, in

which a splicing cascade mediated by Sex-lethal (Sxl) and Transformer (Tra) promotes the formation of either a female or male isoform of Dsx (Dsx^F or Dsx^M, respectively) (Whitworth, Jimenez et al. 2012). Although female forms of *Sxl*, *tra* and *dsx* mRNA were not detected in $chinmo^{ST}$ testes (Figure S2.3A), changes in Dsx^M protein expression were observed. Dsx^M is absent from the ovary (Figure S2.3B) but present in the somatic cells of the testis including hub cells, CySCs and cyst cells (Hempel and Oliver 2007) (Figure 2.7A). Testes from young $chinmo^{ST}$ males closely resemble wild type testes, but a few CySCs and their immediate progeny lack Dsx^M, while older cyst cells retain this male determinant. As somatic cell aggregates and follicle-like cells arise, they all lack Dsx^M , and the remaining older Dsx^M-positive somatic cells become displaced from the testis apex (Figures 2.7B-D). Similar results were obtained in testes with *chinmo* RNAi knockdown in the CySC lineage (data not shown). Although Dsx^M protein is decreased in the CySC lineage in $chinmo^{ST}$ testes, a decrease in dsx^M mRNA is not detectable at the whole testis level (Figure S2.3A), suggesting that a decrease in dsx^M mRNA in CySCs and cyst cells might be masked by the presence of dsx^M mRNA from other cells in the testis, such as sheath, basal epithelial, and seminal vesicle cells. Therefore, we used *dsx-Gal4* to detect changes in *dsx* transcription levels specifically in CySC lineage cells (Robinett, Vaughan et al. 2010). Two independent *dsx-Gal4* lines are active in CySC lineage cells in control testes (Figure 2.7E, G) but not in follicle cells in normal ovaries (Figure S2.3C-D). In $chinmo^{ST}$ testes, however, somatic aggregates and follicle-like cells no longer express these transgenic reporters (Figure 2.7F, H). These results suggest that Chinmo either directly or indirectly regulates dsx^M transcription levels. Since Dsx^M directly represses *Yp1* expression (Burtis, Coschigano et al. 1991, Coschigano and Wensink 1993), the appearance of ectopic *Yp1* transcripts in $chinmo^{ST}$ testes likely reflects loss of Dsx^M (Figure

2.3J). Taken together, these results suggest that Chinmo is required for maintenance of *dsx*^{M} expression in the CySC lineage, and that loss of Dsx^{M} contributes to the male-to-female somatic sex transformation in *chinmo*ST testes. In support of this hypothesis, expression of Dsx^{M} in the CySC lineage in *chinmo-RNAi* testes partially rescues the phenotype (Table 2.1). We have also found, using two independent *dsx-RNAi* lines, that knockdown of *dsx* in the CySC lineage partially phenocopies the *chinmo* mutant phenotype. In most *dsx-RNAi* testes, germ cells overproliferate and arrest at early spermatogonial stages (Figure 2.7J, Figures S2.3I-J) as do germ cells in *chinmo*ST testes (Figure 2.1R), consistent with a mismatch of germline and somatic sex. Some testes also contain small aggregates of somatic cells (Figure S2.3J, arrows) that resemble the follicle-like cells in *chinmo* mutant testes, but they never develop a full layer of follicle-like cells. Together, these results suggest that Chinmo has targets in addition to Dsx^{M} that maintain other aspects of CySC fate and prevent them from transforming into follicle stem cell-like cells. We conclude that male sexual identity is actively maintained in the CySC lineage of the adult testis, and that this requires the concerted action of Chinmo and Dsx^{M}.

Discussion

The male-to-female sex transformation phenotype that we observe in *Drosophila* testes with reduced Chinmo demonstrates that adult somatic stem cells actively maintain their sexual identity in a cell autonomous manner (Figure 2.7K). In vertebrates, sexual phenotypes were thought to be determined exclusively by systemic hormones, but recent studies have found exceptions to this rule. In birds, for example, somatic sexual identity may be largely cell autonomous, as illustrated by lateral gynandromorph chickens; these male:female chimeras have one side that appears male and the other female, even though both sides are exposed to the same blood system and hormone environment (Zhao, McBride et al. 2010). Another example is the adult mouse gonad, where loss of sex-specific transcriptional regulators can cause differentiated somatic cells to transdifferentiate into cells of the opposite sex (Uhlenhaut, Jakob et al. 2009, Matson, Murphy et al. 2011). Other recent work has found that adult stem cells can respond differently to the different levels of hormones found in male and female mice: hematopoietic stem cells, which were thought to function similarly in both sexes, respond to the higher levels of estrogen in females, resulting in differences in self-renewal ability (Nakada, Oguro et al. 2014). We now show, using the *Drosophila* testis as a model, that adult somatic stem cells can also autonomously maintain their sexual identity. Chinmo regulates sexual identity by promoting expression of the male sex determination factor Doublesex^{M} (Dsx^{M}) in CySCs and their progeny. Since *dsx*-family members play central roles in the maintenance of somatic sexual identity in diverse organisms including mice, fish and amphibians (Shibata, Takase et al. 2002, Matson, Murphy et al. 2011, Masuyama and Matsuda 2012), but their upstream regulators are not well understood, it will be interesting to determine whether Chinmo is required for sex maintenance at other times in development and in other

tissues in *Drosophila*, and whether Chinmo homologues function more broadly in sex maintenance in other organisms.

Our data suggest that Chinmo is the central regulator of CySC sex maintenance and implicate Dsx^M is an important target of Chinmo in this process. However, since expression of Dsx^M only partially rescues the sex maintenance phenotype in *chinmo* mutant testes, and *dsx* knockdown only partially phenocopies the *chinmo* mutant phenotype, Dsx^M is unlikely to be the only target of Chinmo. Together, our data support a model in which Chinmo promotes expression of Dsx^M, which in turn regulates its own set of target genes that are critical for male sex identity. In addition to promoting Dsx^M expression, Chinmo likely affects the expression of Dsx-independent target genes that are important for additional aspects of CySC identity. In this case, resupplying Dsx^M does not fully rescue the *chinmo*-RNAi phenotype because these testes still lack Chinmo targets that are independent of Dsx but required for CySC self-renewal. Similarly, CySCs lacking Dsx may only partially recapitulate the Chinmo loss-of-function phenotype because they lack expression of male determinants downstream of Dsx. However, expression of Dsx-independent Chinmo targets may permit maintenance of CySCs lacking Dsx, and may also prevent the complete conversion of these mutant CySCs into Follicle Stem or progenitor-like cells. It is also possible that additional sex determination factors, such as the female determinant Dsx^F, will be required for a full conversion of CySCs lacking Dsx^M into follicle stem or progenitor-like cells. Assessing the role of this and other sex determination pathway members in sex maintenance will be of interest. Furthermore, since our data suggest that Chinmo regulates Dsx^M at the level of transcription, comparison of targets of Chinmo and Dsx^M in the CySC lineage should be informative.

It is intriguing that removing *chinmo* clonally in a few CySCs leads to rapid loss of the mutant CySCs (Flaherty, Salis et al. 2010), while depleting *chinmo* from all CySCs (via RNAi or the $chinmo^{ST}$ mutation) allows them to remain but become feminized. We hypothesize that CySC clones lacking *chinmo* (either weak or strong alleles) are out-competed by wild type CySCs, and that the full sex transformation phenotype only develops when the possibility of competition is removed. (i.e., when most or all CySCs lack *chinmo*). Individual CySCs lacking Chinmo may also undergo sex transdifferentiation, but we have not been able to determine whether or not this is the case, since such CySCs are lost quite rapidly. Comparing testes that contain either a few or many CySCs that lack *chinmo* could be informative, as it should reveal the number of stem cells that must be mutant in order to allow the sex transformation of the testis soma to occur. The ability of stem cells to compete for niche access is a poorly understood yet important aspect of adult stem cell biology in general (Stine and Matunis 2013), and this work provides an avenue for studying the underlying mechanisms.

Our finding that stem cells undergo sexual transformation may provide unique insight into how transdifferentiation is regulated at a cellular and molecular level more generally. Sex transformation of stem cells in adult *Drosophila* testes provides a highly tractable genetic system to study cellular mechanisms by which highly conserved *dsx*-related transcription factors and their target genes actively maintain somatic sexual identity. This may provide insight into human testicular cancers, such as granulosa cell tumors, that may be linked to altered somatic sexual identity (Hanson and Ambaye 2011). Analysis of Chinmo and Dsx/Dmrt1-mediated sex maintenance pathways in somatic stem cells may also yield insight

into the maintenance of sexual dimorphism in other organs, such as the mammalian liver, and provides a unique model to study adult stem cell transdifferentiation *in vivo*.

Experimental Procedures

Fly stocks and cultures

Fly stocks were raised at 25 ˚C on standard molasses/yeast medium unless otherwise indicated. The following fly stocks were used: *UAS-FL-chinmo* and *chinmo*^{1} (Zhu et al., 2006), *eyaA3-Gal4* (Leatherman and DiNardo, 2008), *M5-4* (Gönczy and DiNardo, 1996), *c587-Gal4* (Kai and Spradling, 2003), *nanos-Gal4-VP16* (Van Doren et al., 1998), *tj-Gal4* (Drosophila Genetic Resource Center), *UAS-dsx*^{M} (Lee et al., 2002), and *UAS-lifactin-GFP* and *slbo-lifactin-GFP* (from X. Wang and D. Montell), *UAS-dsx RNAi-1* (*P[KK111266]VIE-260B*, From Vienna Drosophila RNAi Center (VDRC)), *UAS-dsx RNAi-2* (from T. Shirangi and M. Mckeown), *dsx-Gal4-1* (*w*^{1118}*; P[GMR40A05-Gal4]attP2*, From Bloomington Drosophila Stock Center (BDSC)), *dsx-Gal4-2* (*w*$^{-}$*; dsx-Gal4/TM3,Sb,Ser, twist-gal4, UAS-*2xEGFP, from B. Baker). *y w* and *chinmo*ST*/CyO* flies were used as control flies. Other fly stocks were from the VDRC or BDSC including the *SOD[x39]/TM3* line from which *chinmo*ST was isolated.

Immunostaining

Testes and ovaries were dissected, fixed, and stained as described previously (Matunis, Tran et al. 1997). Tyramide signal amplification (Invitrogen) was used to increase sensitivity of rat anti-Dsx^{M} (from B. Oliver, 1:500 dilution). The following antibodies were also used: rabbit anti-Vasa (d-260) and goat anti-Vasa (dN-13) (Santa Cruz Biotechnology, 1:400); rabbit anti-GFP (Torrey Pines Biolabs, 1:10,000); chicken anti-GFP (Abcam, 1:10,000); mouse anti-β-Galactosidase (Promega, 1:1000); mouse 1B1 (1:25), mouse anti-Fasciclin III (1:50), mouse anti-Armadillo (1:50), mouse anti-Eya 10H6 (1:50), and mouse anti-Cut (1:20) (all from

Developmental Studies Hybridoma Bank, University of Iowa); rat-anti-Chinmo (from N. Sokol, 1:500); rabbit anti-ZFH1 (from R. Lehmann, 1:5000); guinea pig anti-Tj (from D. Godt, 1:4000); mouse anti-phospho-Histone H3 (Cell Signaling, 1:200); and rat anti-BrdU MCA2060 (Serotec, 1:40). Alexa fluor-conjugated secondary IgG (H+L) antibodies were diluted at 1:200 for 568 and 633 conjugates and 1:400 for 488 conjugates. Secondary antisera were: goat anti-rat 488, goat anti-rabbit 488 and 568, goat anti-mouse 488, 568 and 633, goat anti-chick 488, and goat anti guinea-pig 568 (Molecular Probes/Invitrogen). DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1 mg/ml.

Lineage analysis

For lineage analysis, *chinmoST; actin>stop>lacZ* or *chinmo^{KG05386}; actin>stop>lacZ* males were mated to *E132-Gal4; chinmoST; UAS-FLP* or *c587-Gal4; chinmoST; UAS-FLP* females to permanently mark hub cells or the CySC lineage, respectively. Newly eclosed flies were then aged prior to testis dissection and immunostaining. To determine whether the *c587-Gal4* and *E132-Gal4* drivers become re-expressed in follicle-like cells in aged *chinmoST* testes, *chinmoST; UAS-lifactin-GFP* males were crossed to *E132-Gal4; chinmoST; tub-Gal80[ts]* or *c587-Gal4; chinmoST; tub-Gal80[ts]* females at 18 ˚C. Newly eclosed progeny were aged at 18 ˚C for 1 week to allow development of the follicle-like cell phenotype, and then switched to 29 ˚C to assess Gal4 activity in chinmoST testes.

mRNA extraction and PCR

mRNA extraction and reverse transcription-PCR were performed as previously described (Issigonis and Matunis 2012) with the following primers (Amrein and Maniatls 1994, Deshpande and Schedl 1999, Tarone, Nasser et al. 2005, Graham and Schedl 2011):

Gene	**Forward primer 5' to 3'**	**Reverse primer 5' to 3'**
Sxl	CGCTGCGAGTCCATTTCC	GTGGTTATCCCCCATATGGC
Tra	GGTCACACTGAGGAAAGTGC	CTTCTCACCCGATCCTGTTCTC
dsx^F	TTCCGCTATCCTTGGGAGC	ACAGAGGTTTTGCTCCATAA
dsx^M	TTCCGCTATCCTTGGGAGC	AAGTGCGCCCCATAGCGACC
Yp	TGAGCGTCTGGAGAACATGAA	GCGACAGGTGGTAGACTTGCT
GAPDH	CAACAATAACAAAATATGGCGGATA	CTATGGCCGAACCCCAGTT
Act5C	GTATCCTCACCCTGAAGTAC	CATGATGGAGTTGTAGGTGG

GAPDH and Act-5C were used as controls.

In vivo BrdU incorporation

Groups of about 20 age-matched adult males were incubated at 25 ˚C in empty vials for 4 hours, and then transferred to vials of fly food overlaid with disks of filter paper soaked with 120 μl of 2.5 mM 5-bromo-2'-deoxyuridine (BrdU) (Life Techniologies) and 5 μl of green food coloring (McCormick). After 24 hours, flies that had not eaten the BrdU (and therefore did not have green guts) were discarded, and BrdU was detected in testes from the remaining flies as described (Brawley and Matunis 2004).

Quantification of cell division and severity of the chinmoST phenotype

CySC mitoses were quantified by counting the number of PH3^{+} Vasa^{-} cells within two cell diameters of the hub. Cyst cell mitoses were quantified by counting the number of PH3^{+} Vasa^{-} cells more than two cell diameters from the hub. To categorize the severity of the *chinmoST* phenotype, we used the following criteria. Morphologically wild-type testes were scored as normal. Testes with epithelial aggregates and/or follicle-like cells at the apex near the hub, but not away from the apex, were scored as having a mild phenotype; these testes often contained overproliferating germ cells. Testes with epithelial aggregates and/or follicle-like cells throughout the entire testis were scored as having a severe phenotype; germ cells in these testes were often arresting at early spermatogonial stage or degenerating. The severity of the *chinmoST* and chinmo RNAi phenotypes both progress with age. Therefore, the presence of a mild vs. severe phenotype was used to estimate testis age when not clearly indicated.

Microscopy and Image Analysis

Fixed testes were mounted in Vectashield (Vector Labs), imaged with a Zeiss LSM 5 Pascal or LSM 510 Meta, and analyzed using the Zeiss LSM Image Browser software; panels are single confocal sections unless stated otherwise. Statistical analysis of pH3 positive cell number was performed with GraphPad Prism 5 software and averages were compared using a two-tailed Student's t test assuming unequal variances. Percentages were analyzed for statistical significance using a chi-squared test.

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Figure Legends

Figure 2.1. Reduction of Chinmo causes somatic cells in adult testes to be gradually replaced by cells resembling ovarian follicle cells.

(**A**) Illustration of a wild-type *Drosophila* testis (right) with the apex magnified (left). Germline stem cells (GSCs, dark green) and somatic cyst stem cells (CySCs, dark blue) adhere to the hub (yellow). GSCs produce differentiating male germ cells (spermatogonia and spermatocytes, green) that are displaced from the hub and form elongated spermatids (grey) and mature sperm (not shown). Approximately two CySCs flank each GSC; CySCs produce squamous, quiescent cyst cells (light blue), which encase differentiating germ cells. **(B)** Illustration of a wild-type *Drosophila* ovariole (top) comprised of a germarium (magnified, bottom) followed by a series of developing egg chambers. In the germarium, anterior niche cells cap cells (grey) support GSCs (dark pink), which produce differentiating female germ cells (light pink). Two somatic follicle stem cells (red), located near the middle of the germarium, produce follicle precursor cells (magenta), which differentiate into follicle cells (orange), stalk cells (purple), and polar cells (yellow). Each egg chamber contains 16 germ cells surrounded by a monolayer of columnar epithelial follicle cells. Polar cells are located at each end; egg chambers are linked by chains of stalk cells. (**C-S**) Immunofluorescence detection in adult testes and ovaries of Tj (C-H, green) to visualize somatic cell nuclei, or FasIII (I-S, green) to highlight the hub in all testes (yellow arrows), and somatic cell membranes in ovaries and *chinmo* mutant testes (arrowheads). Panels I'-N' show the FasIII signal alone. Vasa (red) marks germ cells and DAPI (blue) marks nuclei in all panels. In control testes (C, I, O), somatic CySC lineage cells (arrowheads) are squamous and interspersed among germ cells. Insets (C, I) show GSCs (white arrows) and CySCs (open

arrowheads) surrounding the hub. In *chinmo*ST testes (D-G, J-M, P-R), a distinct phenotype develops over time. Testes from young mutant males (D-E, J-K, P-Q) resemble those from controls except that most (~77%, n = 61) contain aggregates of 8 or more somatic cells (arrowheads); these always appear near the hub (yellow arrows). As flies age (F-G, L-M, R), aggregates expand beyond the testis apex and become columnar and peripheral (arrowheads) in 82% of testes (n = 545), forming FasIII-positive "follicle-like cells" that resemble somatic follicle cells (arrowheads) in control ovaries (H, N, S). Follicle-like cells occasionally invaginate (G, M, white arrows) to envelop groups of germ cells. 1B1 (O-R, green) marks fusomes; branching fusomes in older germ cells in *chinmo*ST testes indicate spermatogonial arrest (R, open arrowheads). Scale bars = 20 μm. See also Figure S2.1.

Figure 2.2: Genetic complementation and rescue experiments indicate that *chinmo*ST is a partial loss-of-function allele of *chinmo*

(**A**) Immunofluorescence detection of FasIII (green at cell periphery) to visualize follicle-like cells in testes, and Vasa (red) to visualize germ cells. Testes from *chinmo*ST / *chinmo*^{M33} males resemble *chinmo*ST homozygous testes (compare to Figure 1R). (**B-C**) The follicle-like cell phenotype in *chinmo*ST testes (B) can be rescued by overexpressing *chinmo* in the CySC lineage (*c587-Gal4; chinmo*ST*; UAS-chinmo/tub-Gal80*^{ts}) (C). The position of the hub is marked with an asterisk. (**D-H**) Immunofluorescence detection of Chinmo (green) in testes and ovaries. In control testes (D), Chinmo is found in the nuclei of hub cells (asterisk), CySC lineage cells (arrowhead), and germ cells (Vasa, red) (Flaherty, Salis et al. 2010). In both young and old *chinmo*ST testes (E-G), Chinmo is present in hub cells and germ cells but is absent from CySC lineage cells (arrowhead). Chinmo is absent from follicle cells (arrowhead)

in control ovaries (H). DAPI marks nuclei (blue). Hubs marked by asterisk. Scale bars = 20 μm. See also Figure S2.1, Table S2.1, Table S2.2.

Figure 2.3. Reduction of Chinmo in adult testes causes somatic stem cells and their progeny to acquire female somatic markers.

(**A-C**) Immunofluorescence detection of Zfh-1 (green) and Castor (red) in testes and ovaries. Insets, red channel only shown in grayscale. In control testes (A), Zfh-1 marks CySCs (arrowhead) and early cyst cells (arrow), but Castor is not detected. In testes from young (1-3 day) *chinmo*ST males (B), some CySCs (arrowheads) and cyst cells (arrow) express both Zfh-1 and Castor, as do ovarian follicle stem cells (solid arrowhead) and their early progeny (follicle cell progenitors, open arrowhead) in control ovaries (C). (**D-F**) Immunofluorescence detection of Cut (green) and Vasa (red). Insets, green channel only, magnified. In control testes (D), Cut is absent from all cells including CySCs (arrowhead), but it is present in follicle-like cells in *chinmo*ST testes (E, arrowhead) and follicle cells in control ovaries (F, arrowhead). (**G-I**) Immunofluorescence detection of *slbo*-GFP (green). *slbo*-GFP is not expressed in control testes (G) but marks later somatic follicle cells (around stage 10) in control ovaries (I). In *chinmo*ST testes (H), *slbo-GFP* marks a subset of follicle-like cells (arrowhead); adjacent follicle-like cells (inset, arrow) are not marked. (**J**) *Yolk protein 1* (*YP1*) expression is detected by RT-PCR in ovaries (O) and testes (T) from 7-9 day *chinmo*ST and *chinmo*^{KG} adults but not from control adults. GAPDH is used as a control. (**K**) Bar graph showing the number of CySCs and cyst cells containing the mitotic marker phospho-histone H3 (PH3) in testes from 1-3 day old control or *chinmo*ST flies. Somatic cells within 2 cell diameters of the hub were scored as CySCs; those more than 2 cell diameters away were scored as cyst cells.

Significantly more mitotic cyst cells are found in *chinmo*ST testes than in control testes (*, p=0.0168). There is no significant difference in the number of mitotic CySCs between *chinmo*ST and control testes. Data represented as mean ± standard error of the mean. (**L-O)** Immunofluorescence detection of PH3 (green in nuclei). FasIII (green) marks cell membranes in the hub (asterisk, L-M), follicle-like cells (N), and follicle cells (O). In control testes (L), PH3 is detected in CySCs (arrowhead); cyst cells away from the hub are non-mitotic and do not express PH3. Cyst cells in young *chinmo*ST testes (M) and follicle-like cells in older *chinmo*ST testes (N) express PH3 (arrowheads), as do follicle cells (arrowheads) in control ovaries (O). Hubs marked by dashed line (A-B) or asterisk (D-E, L-M). DAPI marks nuclei (blue, all panels). Scale bars = 20 μm.

Figure 2.4. Ovarian stalk cell markers are expressed in a subset of somatic cells in *chinmo*ST testes.

(**A-B**) Immunofluorescence detection of Arm (white) and Zfh-1 (green) reveals stalk-like cells in *chinmo*ST testes. In control testes (A), hub cells (yellow arrow) express high levels of Arm and low levels of Zfh-1, and CySCs and their immediate daughters (arrowhead) express low levels of Arm and high levels of Zfh-1. In all other cyst cells, Arm is low and Zfh-1 is absent. In *chinmo*ST testes (B), Arm is low and Zfh-1 is absent in follicle-like cells lining the periphery (solid arrowhead). Other somatic cells (open arrowheads) form aggregates that resemble ovarian stalk cells morphologically and express high levels of Arm and Zfh-1, which are characteristic of stalk cells (data not shown). These aggregates are typically located just beneath the testis sheath, sandwiched between follicle-like cells and the basement membrane. (**C-F**) Immunofluorescence detection of Castor (red) and Zfh-1 (green) reveals stalk-like cells in *chinmo*ST testes. Castor is absent from control testes (C; also see Fig. 2A). Hubs marked by

yellow arrow. In control ovaries (D), Castor is expressed in follicle stem cells and early follicle cell progenitors (bracket; also see Fig. 2C). After egg chamber formation, Castor is restricted to polar cells and Zfh-1^{+} stalk cells (open arrowhead) and is no longer expressed in main-body follicle cells (solid arrowhead). In *chinmo*ST testes (E-F), Castor is expressed in Zfh-1^{+} cell aggregates at the testis apex that resemble follicle cell progenitors (bracket) and in stalk-like cells (open arrowheads) but not in Zfh-1^{-} follicle-like cells (solid arrowhead). Panel F is an enlargement of the boxed area in panel E. Scale bars = 20 μm.

Figure 2.5. Chinmo is required autonomously in adult CySC lineage cells to prevent their transformation into female soma.

(**A-G**) Immunofluorescence detection of FasIII (green at cell periphery) and the germ cell marker Vasa (red) to visualize the morphology of adult testes after expression of *chinmo*-RNAi in different cell types. Before RNAi induction in the CySC lineage (A), testes appear normal. After RNAi induction in the CySC lineage, testes resemble *chinmo*ST testes: somatic cells initially form aggregates (97% of testes, n = 36/37) (B-C, arrowheads) and then follicle-like cells (76% of testes, n = 68/90) (D). RNAi induction in the CySC lineage with a different Gal4 driver also phenocopies *chinmo*ST testes (E). RNAi induction in germ cells (F) or in hub cells (G) does not phenocopy *chinmo*ST. Hubs marked by asterisk. Scale bars = 20 μm. (**H**) Composite bar graph showing the percentage of testes with normal, mild, or severe phenotypes after expression of *chinmo*-RNAi with different somatic drivers. Testes remain morphologically wild type after *chinmo*-RNAi expression only in cyst cells (*eyaA3-Gal4* at 25 ˚C), but after expression in both CySCs and cyst cells (*eyaA3-Gal4* at 29 ˚C, *c587-Gal4*, or *tj-Gal4*), testes display a range of *chinmo*ST phenotypes, as defined in Methods. See also Figure S2, Table S3.

Figure 2.6. Follicle-like cells come from the cyst stem cell lineage but not from hub cells.

(**A-E**) Immunofluorescence detection of β-gal (green), which permanently marks either hub cells alone (A-B) or CySC lineage and hub cells (C-E) in *chinmo*ST testes. Somatic cell aggregates and follicle-like cells (B, D-E, arrowheads) are derived from CySC lineage cells (C, arrowhead) but not from hub cells (A-B, arrows) because they express β-gal in testes with marked CySC lineage cells but not in testes with only marked hubs. (**F-H**) Immunofluorescence detection of the thymidine analog bromodeoxyuridine (BrdU, green). Adult males were fed BrdU for 24 hr prior to dissection to label all cells that traversed S-phase during this time. In control (F) and young *chinmo*ST (G) testes, BrdU is not found in any hub cells (arrows), but many germ cells (red) and CySCs (arrowheads) are BrdU^{+}. BrdU is also found in most follicle-like cells in older *chinmo*ST testes (H, bracket). In all panels, DAPI marks nuclei (blue) and Vasa marks germ cells (red). Scale bars = 20 μm. See also Table S4.

Figure 2.7. Chinmo maintains the male identity of adult somatic stem cells through the canonical sex determination pathway.

(**A-D**) Immunofluorescence detection of Dsx^{M} (green) in adult testes. In control testes (A), Dsx^{M} is expressed in the nuclei of hub cells (asterisk), CySCs (arrowhead), and cyst cells (arrow) (n=59 testes). In young *chinmo*ST testes that are still morphologically normal (B), Dsx^{M} levels are low in some CySCs and early cyst cells (arrowhead) but remain high in differentiated cyst cells away from the apex (arrow) (n=35 testes). In *chinmo*ST testes containing somatic cell aggregates (C), Dsx^{M} levels are low in aggregates (arrowhead) but remain high in differentiated cyst cells (arrow) (n=37 testes). In *chinmo*ST testes with follicle-

like cells (D), Dsx^{M} is absent from all follicle-like cells (n=42 testes). (**E-H**) Immunofluorescence detection of cytoplasmic GFP (green) in adult testes to reflect the transcription of *dsx-Gal4* (two different lines). *dsx-Gal4-1* is expressed in control testes (E) at high levels in CySCs (arrowhead) and cyst cells (arrow), and at lower levels in some hub cells (asterisk) (n=15 testes). In $chinmo^{ST}$ testes (F), *dsx-Gal4-1* expression is absent from somatic aggregates and follicle-like cells (arrowhead) but remains high in differentiated cyst cells away from the apex (arrow) (n=19 testes). *dsx-Gal4-2* is expressed in control testes (G) in early cyst cells (arrow) and in some CySCs (arrowhead) but is not detectable in hub cells (asterisk) (n=21 testes). In $chinmo^{ST}$ testes (H), *dsx-Gal4-2* expression is absent from somatic aggregates (arrowhead) but present in some differentiated cyst cells away from the apex (arrow) (n=28 testes). (**I-J**) Immunofluorescence detection of FasIII and 1B1 (green) and DAPI (blue) in adult testes before and after induction of *dsx-RNAi* in the CySC lineage. Panels I' and J' show the blue channel (DAPI) only in grayscale. Before RNAi induction (I), testes look normal, and cells that stain brightly with DAPI (early germ cells and somatic cells) are restricted to the apex of the testis (n = 32 testes). After RNAi induction (29 ˚C for 10 days) (J), testes fill with germ cells that arrest as early spermatogonia, based on the expansion of the bright DAPI zone and presence of branching fusomes (94% of testes, n = 51). In all panels, nuclei are marked with DAPI (blue); germ cells are marked with Vasa (red); Hubs marked by asterisk (A-C, E-G) or dashed line (I-J). scale bars = 20 μm. (**K**) Schematic drawings of a young (left) and older (center) *chinmo* mutant testis apex, and an older *chinmo* mutant testis (right) with the apex indicated (red box). In young *chinmo* mutant testes, CySCs and their early progeny lose male fate and adopt a follicle stem cell/progenitor-like cell identity (magenta). As flies age, follicle stem cell/progenitor-like cells produce follicle-like cells

(orange) and stalk-like cells (purple), which gradually displace normal cyst cells from the niche. The germ cells, which become restricted to the lumen of the tissue, maintain characteristics of their male identity, but over-proliferate and arrest as spermatogonia since the germline and somatic sex are mismatched. See also Figure S2.3.

Figure S2.1. (Related to Figure 2.1 and 2.2) Reduction of Chinmo causes somatic cells in adult testes to be gradually replaced by cells resembling ovarian follicle cells.

(A-C) Immunofluorescence detection of β-galactosidase (green) to visualize M5-4, a male-specific *escargot* enhancer trap, and Vasa (red) to visualize germ cells. In control testes (A), M5-4 is expressed in hub cells (yellow arrow), GSCs and early differentiated germ cells (yellow arrowheads), but not in CySC lineage cells (white arrowhead) or later differentiated germ cells (white arrow). After RNAi-mediated depletion of *chinmo* from the CySC lineage (*c587-Gal4; UAS-chinmo-RNAi*) (B), hub cells and germ cells continue to express M5-4, suggesting that they maintain characteristics of their male identity. Germ cells away from the testis apex fail to downregulate M5-4 (yellow arrowhead), indicating that they do not differentiate properly. M5-4 is absent from ovaries (C). (**D**) Immunofluorescence detection of FasIII (green at cell periphery) to visualize follicle-like cells in testes, and Vasa (red) to visualize germ cells. At 2-3 weeks of age, germ cells in $chinmo^{ST}$ testes degenerate; only somatic cells remain at the testis apex. (**E-F**) Immunofluorescence detection of Chinmo (red) in adult testes containing $chinmo^{1}$ or control clones verifies the specificity of anti-Chinmo antisera in adult testes. Hubs marked by dashed line. Chinmo levels are decreased in a $chinmo^{1}$ germ cell clone (white arrowhead) and somatic clones (white arrows), identified by the absence of GFP (green), compared to neighboring GFP-positive heterozygous germ cells (one

indicated, open arrowhead) and somatic cells (yellow arrow) (E). Wild-type control somatic clones and neighboring cells (white and yellow arrows, respectively) (F) have similar levels of Chinmo. DAPI marks nuclei (blue). Scale bars = 20 μm.

Figure S2.2. (Related to Figure 2.5) Chinmo is required autonomously in adult CySC lineage cells to prevent their transformation into female soma.

(**A-B**) Immunofluorescence detection of nuclear-localized UAS-GFP (green) in adult testes reveals that *c587-Gal4* (A) and *tj-Gal4* (B) are expressed in early CySC lineage cells but not in hub cells (asterisk) or germ cells (red). (**C-D**) Immunofluorescence detection of Chinmo (green) in adult testes verifies the efficacy of *chinmo RNAi* in the CySC lineage (genotype: *c587-Gal4; UAS-chinmo RNAi/tub-Gal80^{ts}*). Before expression of *chinmo* RNAi (C), Chinmo is detected in hub cells (asterisk), CySC lineage cells (arrowheads), and germ cells (red). After 6 days of *chinmo*-RNAi expression in CySC lineage cells (D), Chinmo is no longer detected in CySC lineage cells (arrowhead). (**E-F**) Immunofluorescence detection of GFP (green), revealing the *eyaA3-Gal4* expression pattern. At 25 ˚C (E), *eyaA3-Gal4* drives expression of *UAS-GFP* at high levels in cyst cells (arrow) but not in CySCs (arrowheads). At 29 ˚C (F), *eyaA3-Gal4* drives expression in both CySCs and cyst cells. (**G-I**) Immunofluorescence detection of Chinmo (green) and Tj (red) in adult testes. Hubs marked by dashed line. In control testes (G), Chinmo is detected in CySCs and their immediate daughters (arrows), which are identified as Tj^{+} nuclei near the hub, as well as in cyst cells (arrowheads). After *chinmo*-RNAi is expressed only in cyst cells (*eyaA3-Gal4* at 25 ˚C) (H), Chinmo remains detectable in CySCs and their immediate daughters (arrows) but not in older cyst cells (arrowheads). After *chinmo*-RNAi is expressed in both CySCs and cyst cells (*eyaA3-Gal4* at

29 ˚C) (I), Chinmo is no longer detected in CySCs (arrows) or cyst cells (arrowheads). (**J-L**) Immunofluorescence detection of cytoplasmic GFP (green), revealing the *c587-Gal4* expression pattern. *c587-Gal4* drives expression of *UAS-GFP* in the cytoplasm of early CySC lineage cells in control testes (J) and in somatic cells in the germarium in control ovaries (L). Older follicle cells in control ovaries (L) and follicle-like cells in *chinmo*ST testes (K) lack *c587-Gal4* activity. DAPI marks nuclei (blue in A-E, J-L and white in G-I). Scale bars = 20 μm.

Figure S2.3. (Related to Figure 2.7) *chinmo*ST regulates *dsx* transcription rather than female-specific splicing of mRNAs in the canonical sex determination pathway, and *dsx*-RNAi partially phenocopies the *chinmo* mutant phenotype.

(**A**) RT-PCR detection of male and female spliced forms of *sxl*, *tra* or *dsx* mRNA shows that the female spliced forms are not ectopically expressed in control, *chinmo*ST or *chinmo*^{KG} testes. Actin-5C is used as a control. (**B**) Immunofluorescence detection of Dsx^{M} (green) in control ovaries. Dsx^{M} is not detectable in follicle cells (arrowhead) or any other cells in ovarioles. The green staining outside the ovarioles reflects non-specific staining of the ovarian sheath. (**C-D**) Immunofluorescence detection of GFP (green) in control ovaries to reflect the transcription of *dsx-Gal4* (two different lines). *dsx-Gal4-1* is expressed in escort cells (arrow) but not in follicle cells (arrowhead). *dsx-Gal4-2* is absent from control ovaries, including follicle cells (arrowhead). (**E-F**) Immunofluorescence detection of Dsx^{M} (green) in adult testes before and after induction of *dsx-RNAi* (line 1) in the CySC lineage. Before RNAi induction (E), Dsx^{M} is expressed in the nuclei of hub cells (asterisk) and CySCs lineage cells (arrow) (n=18 testes). After RNAi induction (F), Dsx^{M} levels are low or absent in most CySCs and cyst cells (arrow)

but remain high in hub cells (n=25 testes). (**G**) Composite bar graph showing the percentage of testes with normal, mild, or severe phenotypes after expression of *dsx-RNAi* (line 1) in the CySC lineage in adult testes for the number of days indicated (genotype: *c587-Gal4; UAS-dsx-RNAi-1; tub-Gal80^{ts}*). In this experiment, morphologically wild type testes were scored as normal; testes with somatic cell aggregates and/or over-proliferating early germ cells at the apex, but not away from the apex, were scored as having a mild phenotype; and testes with over-proliferating early germ cells throughout the entire testis were scored as having a severe phenotype (see Fig. 6J). Before RNAi induction (0 d at 29 ˚C), all testes look normal. After RNAi induction in CySC lineage cells, testes display a range of mild to severe phenotypes. (**H-J**) Immunofluorescence detection of FasIII and 1B1 (green) and DAPI (blue) in adult testes before and after induction of *dsx-RNAi* (line 2) in the CySC lineage (genotype: *UAS-dsx-RNAi-2; eyaA3-Gal4; tub-Gal80^{ts}*). Before RNAi induction (H), testes look normal (100% of testes, n =35). After RNAi induction (29 ˚C for 2 weeks) (I, J), germ cells overproliferate and arrest at early spermatogonial stages (72% of testes, n = 39). In addition, these testes contain some aggregates of FasIII^{+} cells at the periphery, which resemble the follicle-like cells in *chinmo-RNAi* testes (J, arrows). Vasa marks germ cells (red) and DAPI marks nuclei (blue). Scale bars = 20 μm.

Table 2.1: Overexpressing DsxM in the CySC lineage rescues Chinmo knock down phenotype

Use c587-Gal4 to express: [1]	% testes with FasIII^{+} somatic aggregates[2]					
	0 d	4 d	7 d	9 d	11 d	14d
UAS-chinmoRNAi-1	0 (n=31)	6.3 (n=64)	73.8 (n=103)	80.5 (n=133)	97.1 (n=35)	100 (n=59)
UAS-chinmoRNAi-1, UAS-DsxM	0 (n=25)	5.3 (n=76)	36.6 (n=153)	55.9 (n=145)	39.4 (n=33)	86.3 (n=124)
UAS-DsxM	0 (n=18)	N/A	0 (n=24)	5.3 (n=19)	0 (n=21)	N/A

[1] *UAS-chinmoRNAi-1 = chinmo^{HM04048}*

[2] All flies were raised at 18 ˚C, which suppresses RNAi induction. After eclosion, adult flies were shifted to 29 ˚C for the indicated amount of time to induce RNAi

[3] For DsxM rescue, $p<0.0001$ at 7, 9, 11 days, $P<0.01$ at 14 days

Table S2.1 (Related to Figure 2.2): Phenotype characterization for combinations of *chinmo* mutant alleles

	chinmoST [1]	*chinmo*^{KG} [2]	*chinmo*^{M33} [3]	*chinmo*^{1} [4]	*Df(chinmo)* [5]	*Other Df* [6]
chinmoST	FC [7]	FC	FC	FC	FC	No FC
chinmo^{KG}	FC	lethal	FC	FC	FC	not tested

[1], [2] *chinmo*ST is a partial loss-of-function allele. It has no mutation in the coding region but has a 10 bp deletion in the 5'UTR region. *chinmo*^{KG} denotes *chinmo*^{KG05386}, which has an 11.5 kb transposable element inserted in the 2nd intron (Flybase). *chinmo*ST and *chinmo*^{KG} are fully viable when crossed to each other or to deficiencies for *chinmo.*

[3], [4] *chinmo*^{M33} encodes a missense mutation (F88I) in the BTB domain (Flybase). *chinmo*^{1} is a protein null allele of *chinmo*. *chinmo*^{M33} and *chinmo*^{1} are lethal when crossed to each other or to deficiencies for *chinmo*, which indicates that they are stronger alleles than *chinmo*ST or *chinmo*^{KG}. *chinmo*^{M33} or *chinmo*^{1} mutant CySC clones are lost from the adult testis stem cell niche (Flaherty, Salis et al. 2010) and no sex transformation is observed. It is possible that CySC clones are lost before the male-to-female transformation phenotype can develop.

[5] Df(*chinmo*) includes the following deficiencies for *chinmo: Df(2L)ast2, Df(2L)Exel6005, Df(2L)dp-79b, Df(2L)S2, Df(2L)ED7762, Df(2L)BSC480,* and *Df(2L)BSC521.*

[6] Other Df includes 58 deficiencies from the Bloomington Stock Center's deficiency kit for the second chromosome that do not remove *chinmo*.

[7] FC = follicle cell-like phenotype.

Table S2.2 (Related to Figure 2.2): The *chinmo*ST phenotype can be rescued by Chinmo overexpression in the CySC lineage

Genotype[1]	% testes with follicle-like cell phenotype
chinmoST (*c587-Gal4; chinmo*ST*; TM6B/tub-Gal80*^{ts})	85.7 (42/49)
chinmoST + Chinmo OE[2, 3] (*c587-Gal4; chinmo*ST*; UAS-FL-chinmo/tub-Gal80*^{ts})	0 (0/47)

[1] Flies were raised and aged for 14-16 days at 18 ˚C.

[2] *UAS-FL-chinmo* (full length *chinmo*) alone, without a Gal4 driver, does not rescue *chinmo*ST.

[3] With a Gal4 driver (*c587-Gal4*), *UAS-FL-chinmo* expresses enough Chinmo at 18 ˚C to rescue *chinmo*ST. Therefore, we cannot use temperature to control the timing of Chinmo overexpression, and we are not able to determine if *chinmo*ST can be rescued by Chinmo overpression only in adult flies, after formation of the follicle-like cell phenotype.

Table S2.3a (Related to Figure 2.5): Chinmo knockdown in CySCs and cyst cells (CC), but not in late cyst cells alone, induces the follicle cell-like phenotype[1]

Express *UAS-chinmo RNAi* with[2]				% testes		
Gal4 Driver	Pattern	RNAi allele[2]	No. of testes	No phenotype	Mild[3] phenotype	Severe[4] phenotype
-	-	1	40	95	5	0
-	-	2	16	100	0	0
c587	CySC, CC	1	59	0	59	41
tj	CySC, CC	1	19	5	42	53
tj	CySC, CC	2	26	31	58	11
Simj[BG00403][5]	Late CC	1	20	100	0	0
CG3964[BG00933][6]	Late CC	1	29	100	0	0
CG42669[BG02427][7]	Late CC	1	12	100	0	0
tai[BG01746][8]	Late CC	1	29	100	0	0

[1] All flies were raised at 18 ˚C, at which temperature RNAi is not induced. After eclosion, adult flies were shifted to 29 ˚C or 31 ˚C for 1-2 weeks to induce RNAi.

[2] *UAS-chinmo RNAi-1 = chinmo^{HM04048}; UAS-chinmo RNAi-2 = chinmo^{HMS00036}.*

[3], [4] See Methods for quantitation of phenotype severity.

[5]-[8] Gal4 enhancer traps that are expressed in testes: *simj[BG00403]* (late cyst cells, pigment cells, muscle sheath), *CG3964[BG00933]* (pigment cells, muscle sheath), *CG42669[BG02427]* (late cyst cells), *tai[BG01746]* (late cyst cells, pigment cells) (Bunt, Monk et al. 2012).

Table S2.3b (Related to Figure 2.5): Chinmo knock down in CySCs and cyst cells induces the follicle cell-like phenotype with time

Express *UAS-chinmoRNAi-1* with[1]				% testes		
Driver	Pattern	Days of RNAi induction[2]	No. of testes	No phenotype	Mild[3] phenotype	Severe[4] phenotype
c587	CySC, CC	0	30	100	0	0
		7-9	37	3	97	0
		14[5]	59	0	59	41
		21-28	90	1	23	76

[1] *UAS-chinmoRNAi-1* = $chinmo^{HM04048}$

[2] All flies were raised at 18 ˚C, at which temperature RNAi is not induced. After eclosion, adult flies were shifted to 29 ˚C for the indicated amount of time to induce RNAi.

[3], [4] See Methods for quantitation of phenotype severity.

[5] These are the same data as in Table S3a (*c587-Gal4>UAS-chinmoRNAi-1*).

Table S2.4 (Related to Figure 2.6): Follicle-like cells arise from the CySC lineage

	% normal testes that have marked CySC lineage cells	% testes with mild phenotype[1] that have marked somatic aggregates	% testes with severe phenotype[2] that have marked follicle-like cells
CySC lineage tracing[3]	100.0 (9/9)	100.0 (72/72)	94.0 (47/50)
Hub lineage tracing[4]	4.5 (4/88)	4.3 (1/23) [5]	6.3 (1/16) [6]

[1], [2] See Methods for quantitation of phenotype severity.

[3] 100% testes have at least some marked CySC lineage cells, but not all CySC lineage cells within each testis are marked. Full Genotype: (*c587-Gal4; chinmoST/chinmo^{KG}; UAS-FLP/Actin>Stop>lacZ*).

[4] 100% testes have at least some marked hub cells, but not all hub cells within each testis are marked. Full genotype: (*E132-Gal4; chinmoST/chinmo^{KG}; UAS-FLP/Actin>Stop>lacZ*).

[5] One testis has one marked cyst cell.

[6] One testis has three marked follicle-like cells.

Figure 2.1

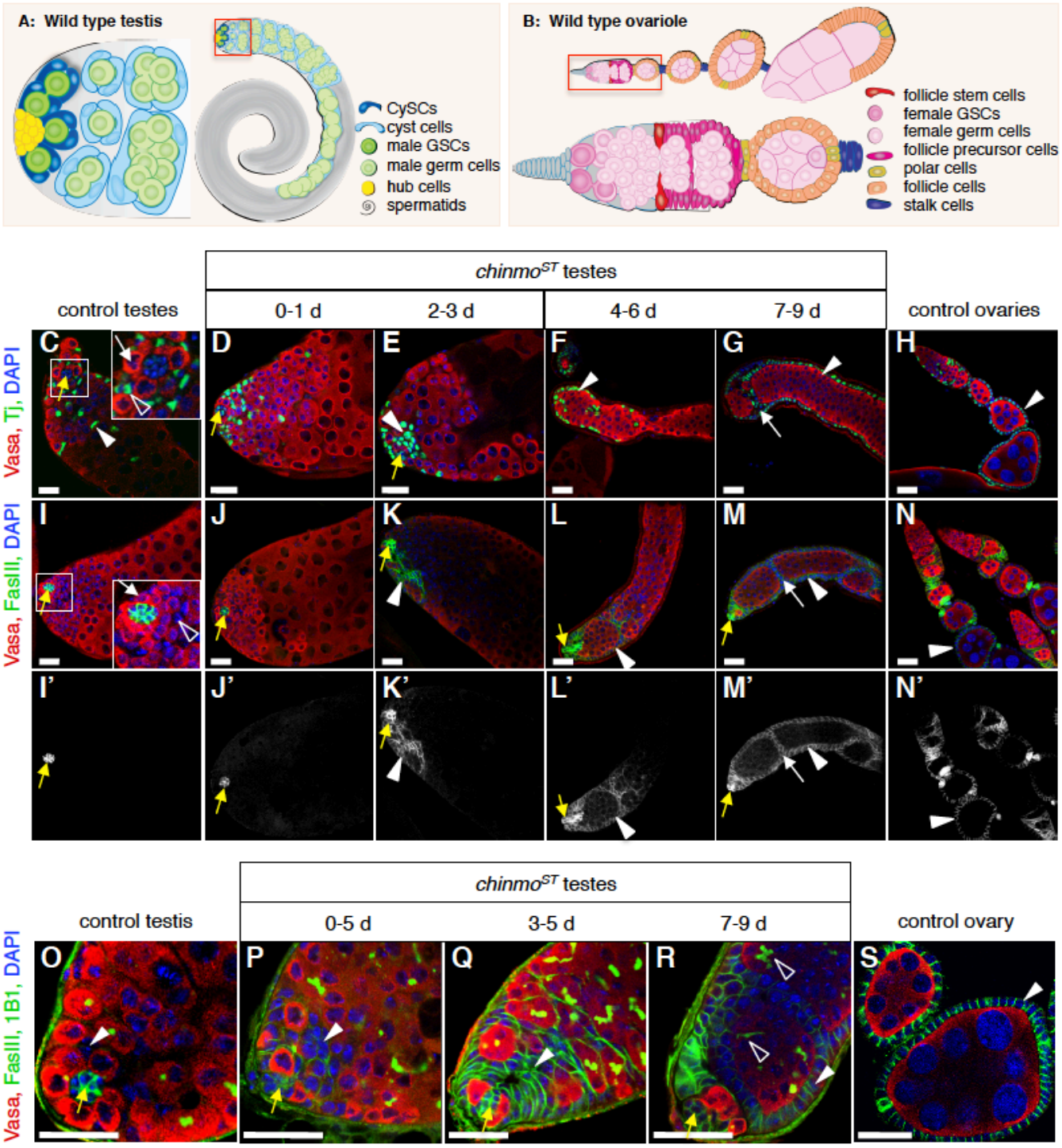

A: Wild type testis
CySCs
cyst cells
male GSCs
male germ cells
hub cells
spermatids
B: Wild type ovariole
follicle stem cells
female GSCs
female germ cells
follicle precursor cells
polar cells
follicle cells
stalk cells
chinmoST testes
control testes
0-1 d
2-3 d
4-6 d
7-9 d
control ovaries
Vasa, Tj, DAPI
Vasa, FasIII, DAPI
C
D
E
F
G
H
I
J
K
L
M
N
I'
J'
K'
L'
M'
N'
chinmoST testes
control testis
0-5 d
3-5 d
7-9 d
control ovary
Vasa, FasIII, 1B1, DAPI
O
P
Q
R
S

Figure 2.2

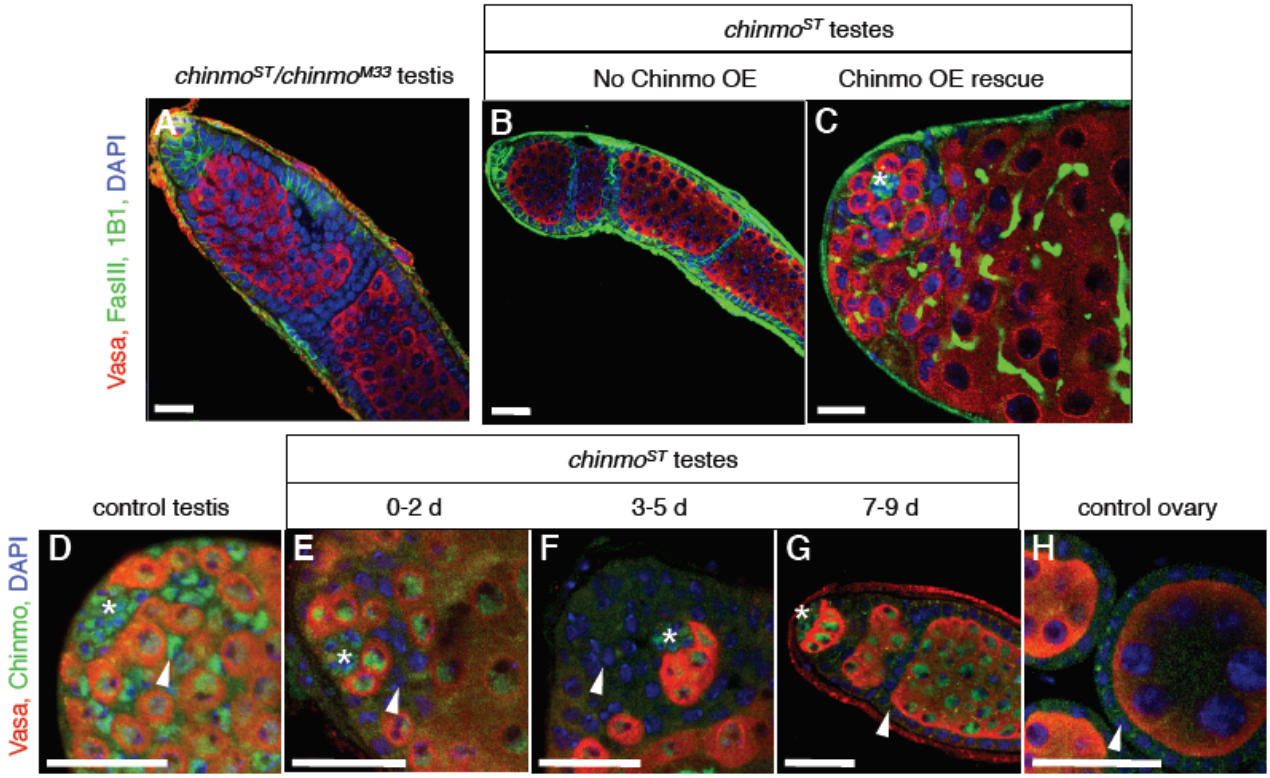

chinmo^ST testes
chinmo^ST/chinmo^M33 testis
No Chinmo OE
Chinmo OE rescue
Vasa, FasIII, 1B1, DAPI
A
B
C
chinmo^ST testes
control testis
0-2 d
3-5 d
7-9 d
control ovary
Vasa, Chinmo, DAPI
D
E
F
G
H

Figure 2.3

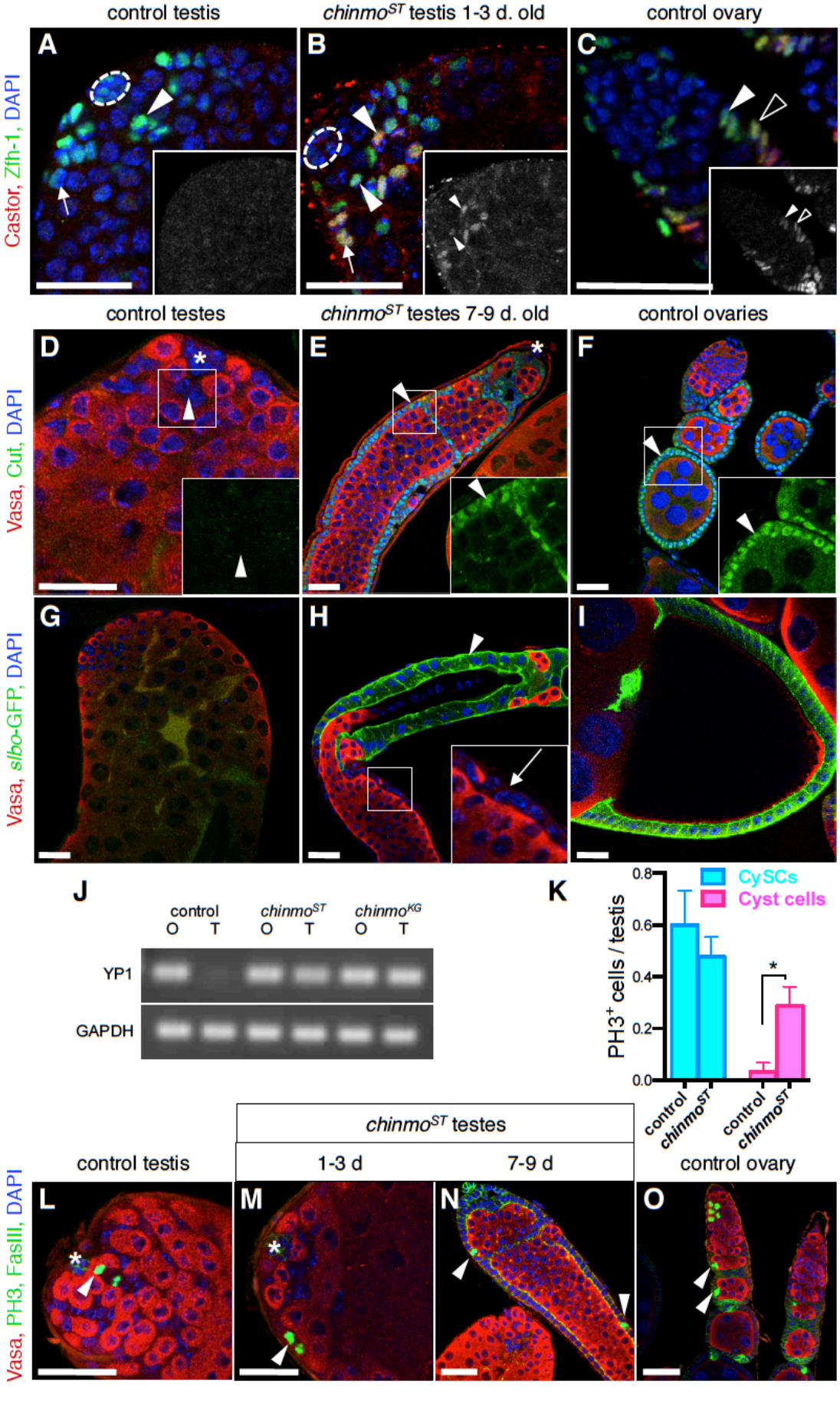

control testis
chinmo^ST testis 1-3 d. old
control ovary
Castor, Zfh-1, DAPI
A
B
C
control testes
chinmo^ST testes 7-9 d. old
control ovaries
Vasa, Cut, DAPI
D
E
F
Vasa, slbo-GFP, DAPI
G
H
I
J
control
chinmo^ST
chinmo^KG
O T O T O T
YP1
GAPDH
K
CySCs
Cyst cells
PH3+ cells / testis
0.8
0.6
0.4
0.2
0.0
control
chinmo^ST
control
chinmo^ST
chinmo^ST testes
control testis
1-3 d
7-9 d
control ovary
Vasa, PH3, FasIII, DAPI
L
M
N
O

Figure 2.4

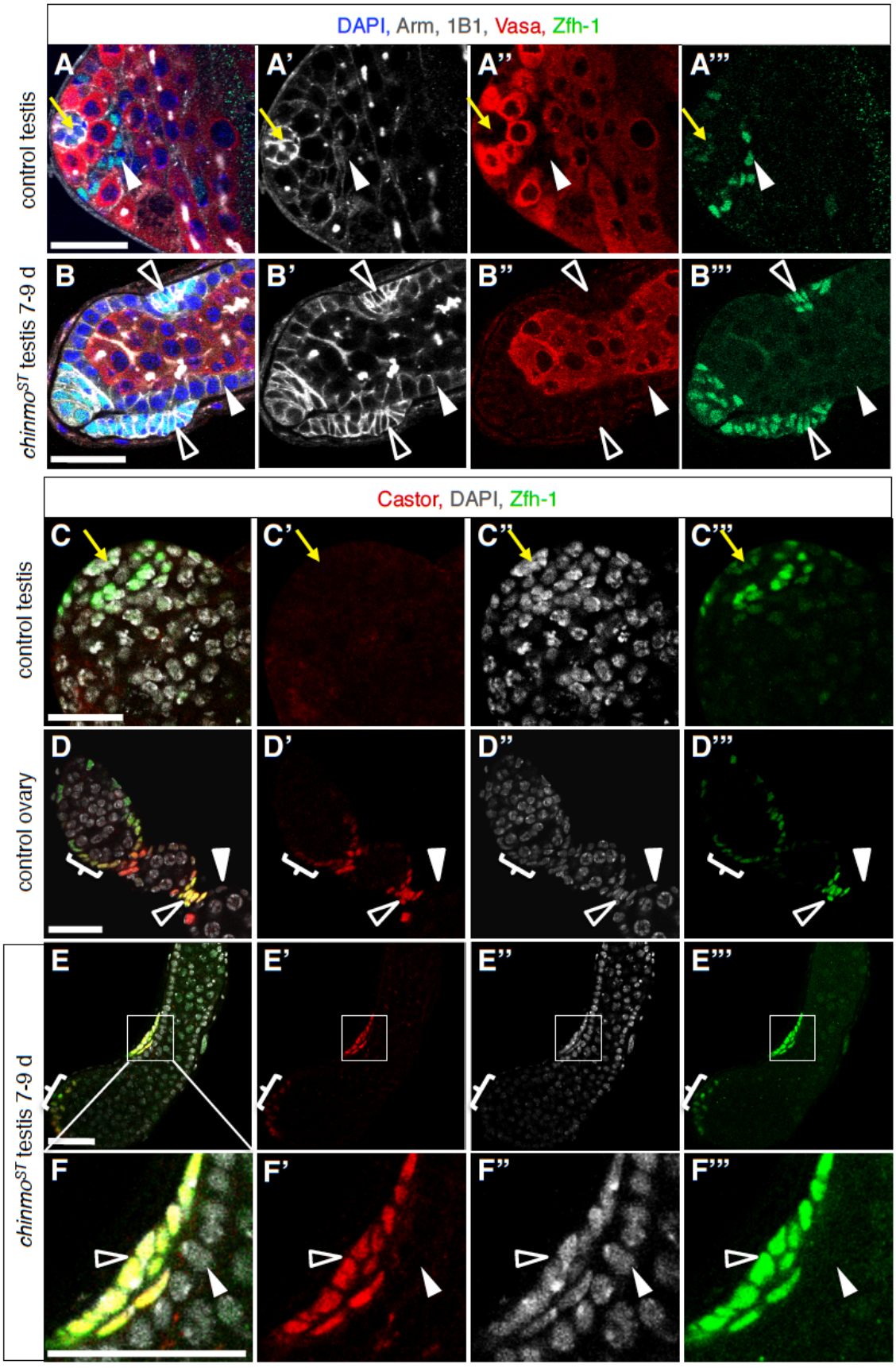

DAPI, Arm, 1B1, Vasa, Zfh-1
control testis
chinmo^ST testis 7-9 d
A
A'
A''
A'''
B
B'
B''
B'''
Castor, DAPI, Zfh-1
control testis
control ovary
chinmo^ST testis 7-9 d
C
C'
C''
C'''
D
D'
D''
D'''
E
E'
E''
E'''
F
F'
F''
F'''

Figure 2.5

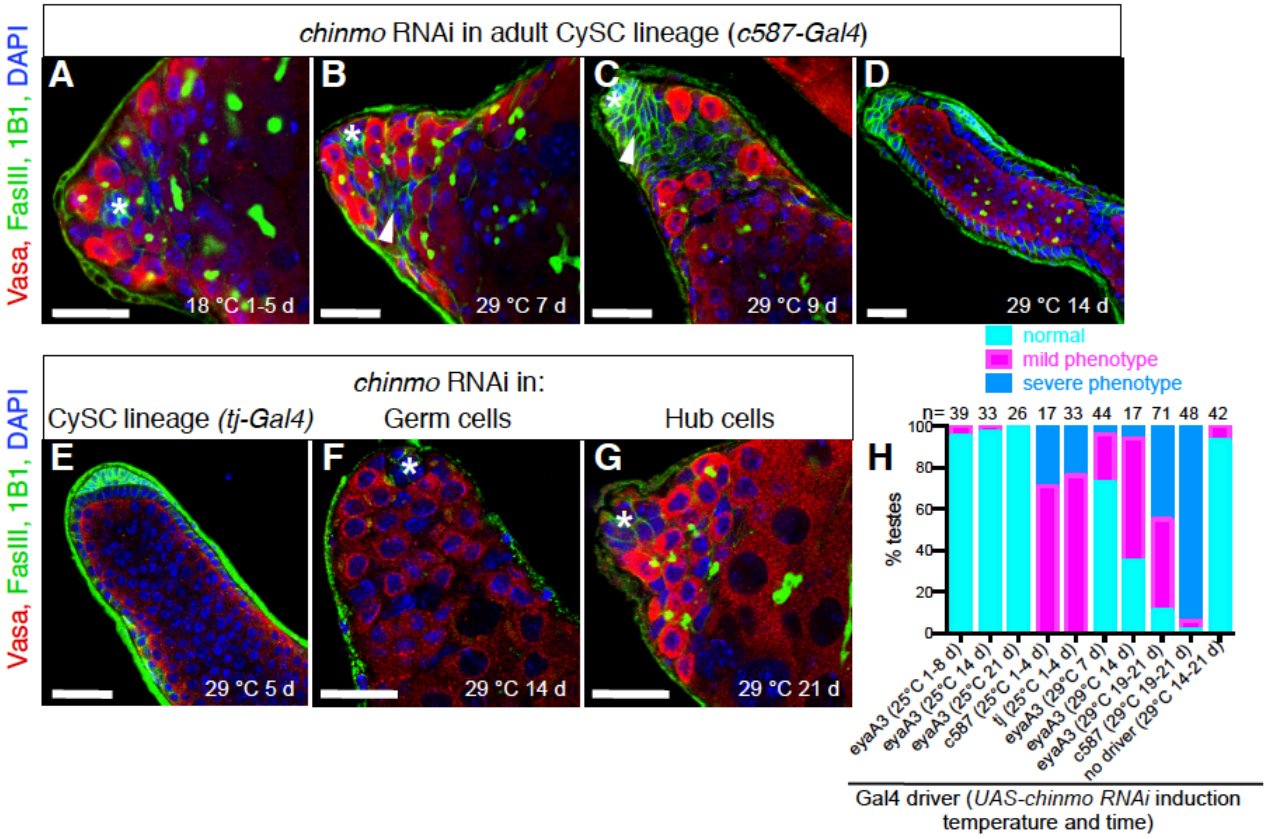

chinmo RNAi in adult CySC lineage (c587-Gal4)
Vasa, FasIII, 1B1, DAPI
A
18 °C 1-5 d
B
29 °C 7 d
C
29 °C 9 d
D
29 °C 14 d
chinmo RNAi in:
CySC lineage (tj-Gal4)
Germ cells
Hub cells
E
29 °C 5 d
F
29 °C 14 d
G
29 °C 21 d
normal
mild phenotype
severe phenotype
H
n= 39 33 26 17 33 44 17 71 48 42
% testes
eyaA3 (25°C 1-8 d)
eyaA3 (25°C 14 d)
eyaA3 (25°C 21 d)
c587 (25°C 1-4 d)
tj (25°C 1-4 d)
eyaA3 (29°C 7 d)
eyaA3 (29°C 14 d)
eyaA3 (29°C 19-21 d)
c587 (29°C 19-21 d)
no driver (29°C 14-21 d)
Gal4 driver (UAS-chinmo RNAi induction temperature and time)

Figure 2.6

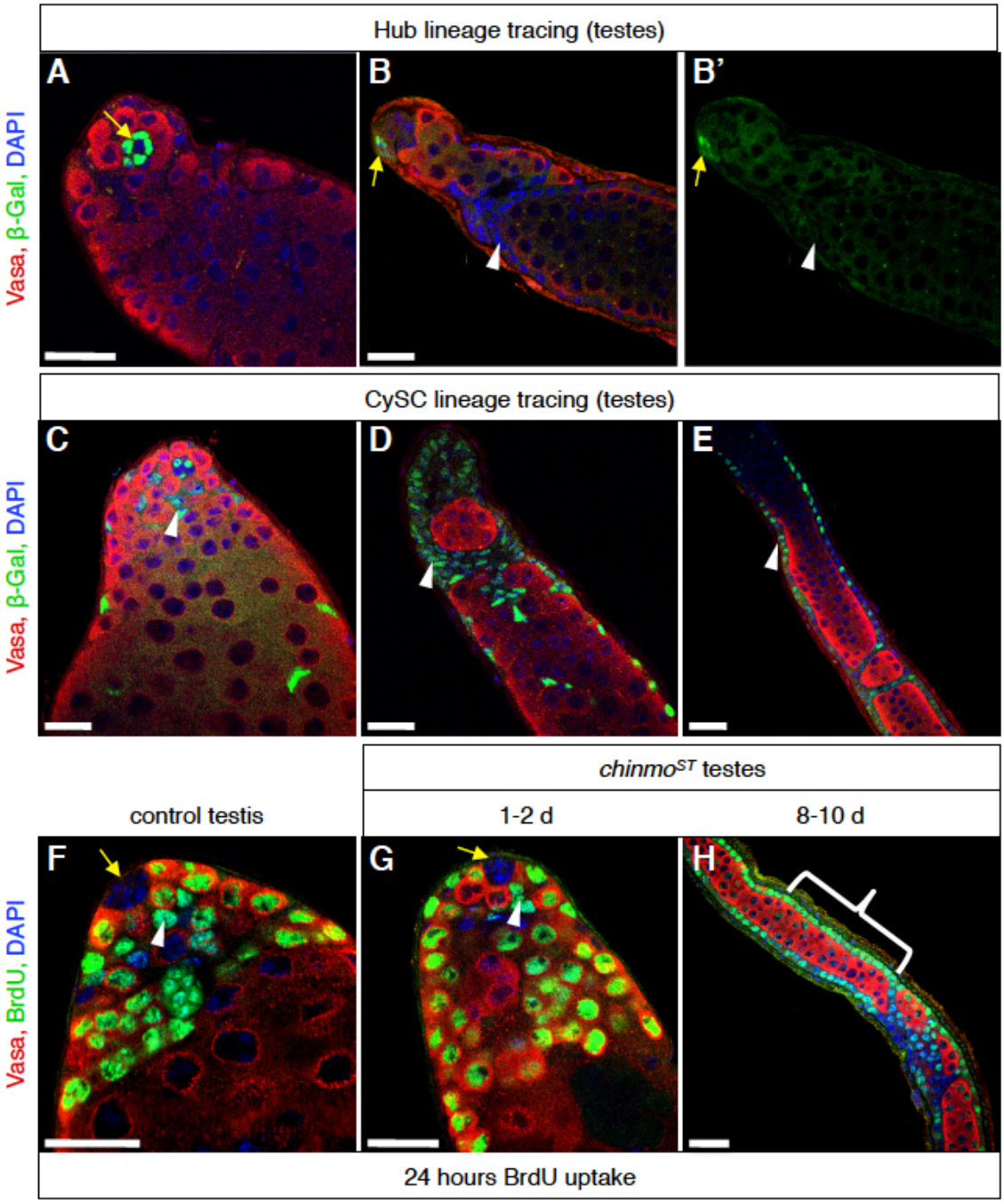

Hub lineage tracing (testes)
A
B
B'
Vasa, β-Gal, DAPI
CySC lineage tracing (testes)
C
D
E
Vasa, β-Gal, DAPI
chinmo^ST testes
control testis
1-2 d
8-10 d
F
G
H
Vasa, BrdU, DAPI
24 hours BrdU uptake

Figure 2.7

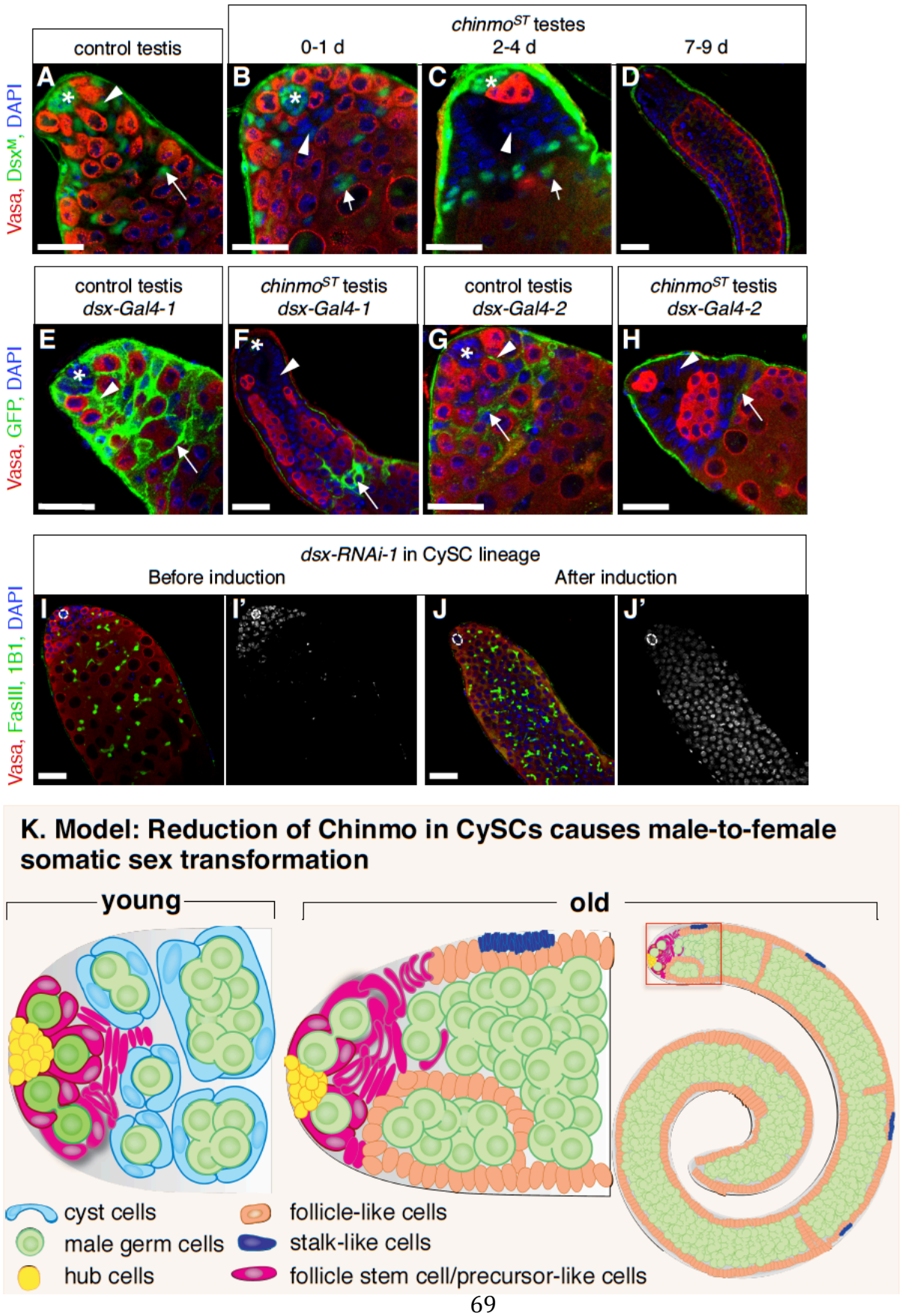

control testis
chinmoST testes
0-1 d
2-4 d
7-9 d
A
B
C
D
Vasa, DsxM, DAPI
control testis
dsx-Gal4-1
chinmoST testis
dsx-Gal4-1
control testis
dsx-Gal4-2
chinmoST testis
dsx-Gal4-2
E
F
G
H
Vasa, GFP, DAPI
dsx-RNAi-1 in CySC lineage
Before induction
After induction
I
I'
J
J'
Vasa, FasIII, 1B1, DAPI
K. Model: Reduction of Chinmo in CySCs causes male-to-female somatic sex transformation
young
old
cyst cells
male germ cells
hub cells
follicle-like cells
stalk-like cells
follicle stem cell/precursor-like cells

Figure S2.1

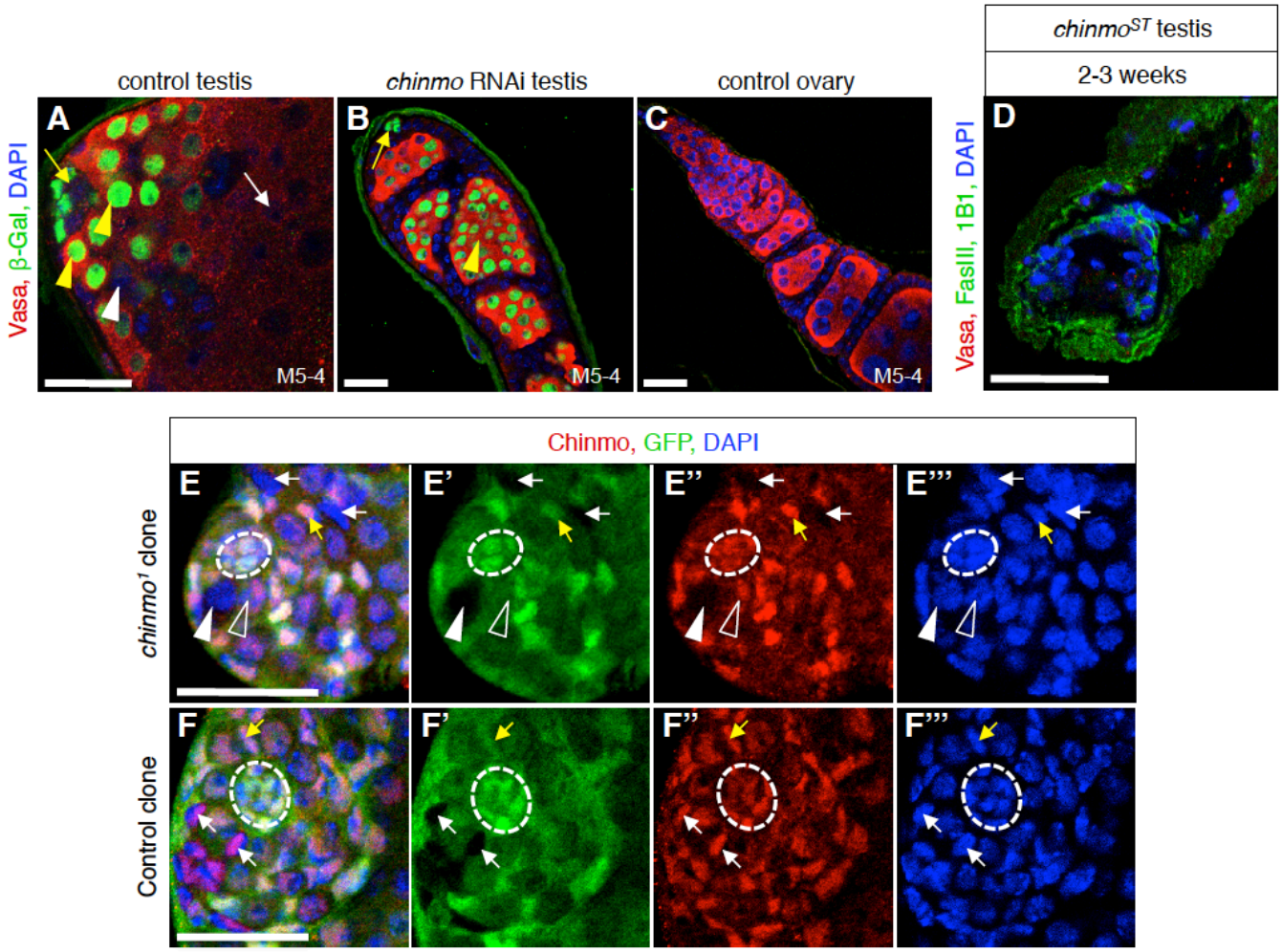

control testis
chinmo RNAi testis
control ovary
chinmoST testis
2-3 weeks
Vasa, β-Gal, DAPI
Vasa, FasIII, 1B1, DAPI
A
B
C
D
M5-4
M5-4
M5-4
Chinmo, GFP, DAPI
chinmo1 clone
Control clone
E
E'
E''
E'''
F
F'
F''
F'''

Figure S2.2

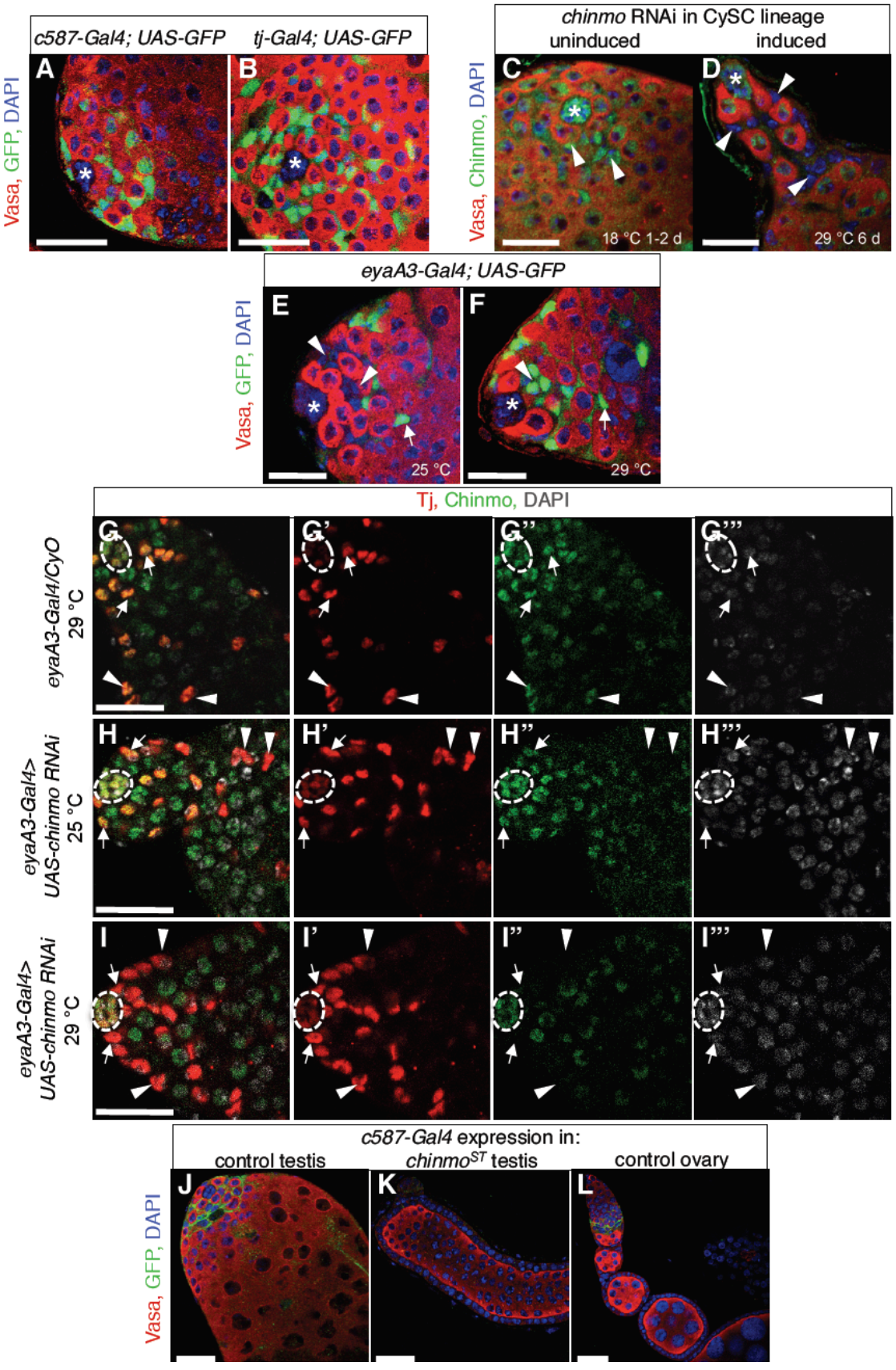
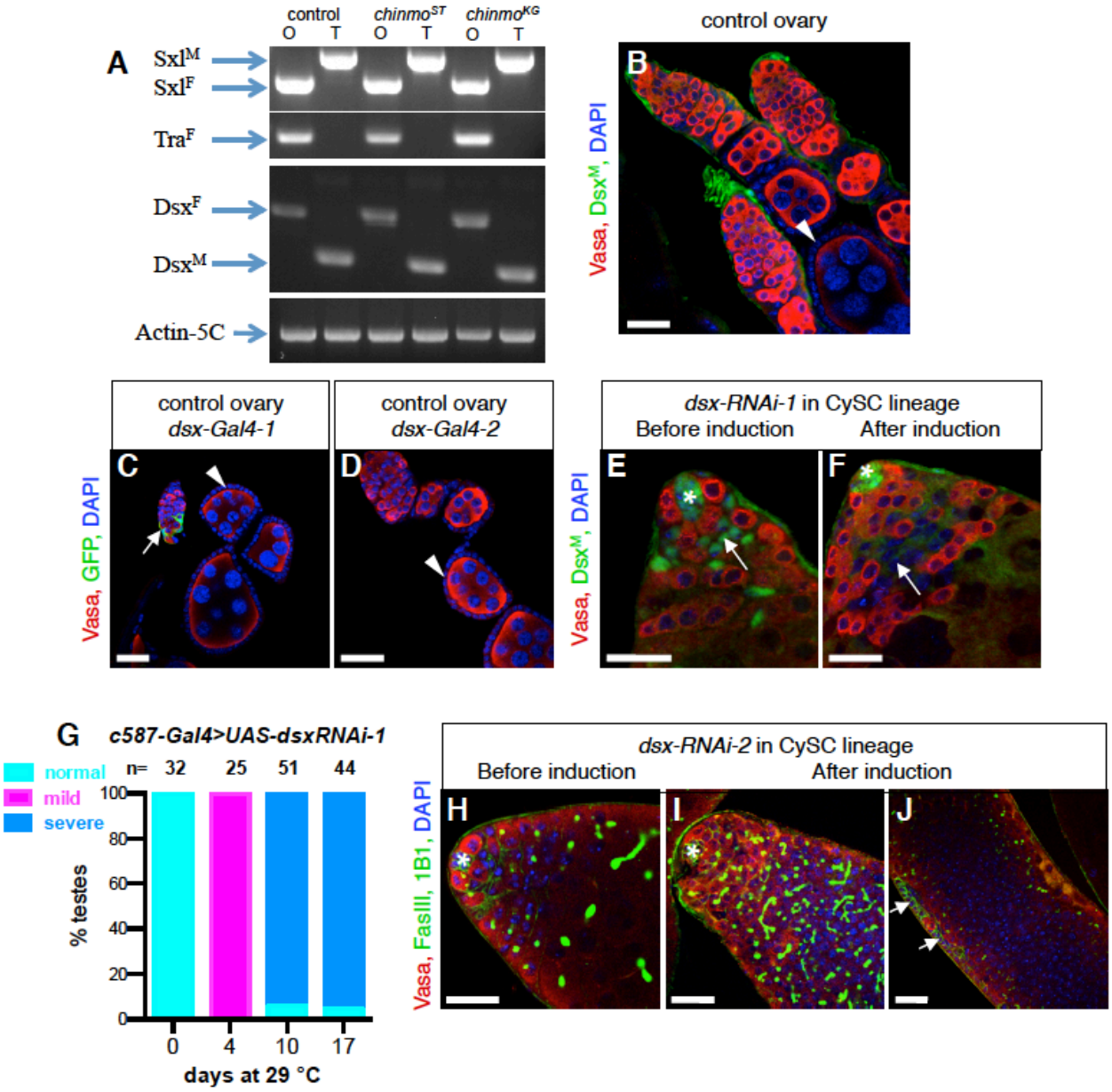

c587-Gal4; UAS-GFP
tj-Gal4; UAS-GFP
Vasa, GFP, DAPI
A
B
chinmo RNAi in CySC lineage
uninduced
induced
Vasa, Chinmo, DAPI
C
D
18 °C 1-2 d
29 °C 6 d
eyaA3-Gal4; UAS-GFP
Vasa, GFP, DAPI
E
F
25 °C
29 °C
Tj, Chinmo, DAPI
eyaA3-Gal4/CyO
29 °C
G
G'
G''
G'''
eyaA3-Gal4>
UAS-chinmo RNAi
25 °C
H
H'
H''
H'''
eyaA3-Gal4>
UAS-chinmo RNAi
29 °C
I
I'
I''
I'''
c587-Gal4 expression in:
control testis
chinmoST testis
control ovary
Vasa, GFP, DAPI
J
K
L

Figure S2.3


A
control
O T
chinmo^ST
O T
chinmo^KG
O T
Sxl^M
Sxl^F
Tra^F
Dsx^F
Dsx^M
Actin-5C
B
control ovary
Vasa, Dsx^M, DAPI
C
control ovary
dsx-Gal4-1
Vasa, GFP, DAPI
D
control ovary
dsx-Gal4-2
E
dsx-RNAi-1 in CySC lineage
Before induction
After induction
Vasa, Dsx^M, DAPI
F
G
c587-Gal4>UAS-dsxRNAi-1
normal
mild
severe
n= 32 25 51 44
% testes
100
80
60
40
20
0
0 4 10 17
days at 29 °C
dsx-RNAi-2 in CySC lineage
Before induction
After induction
Vasa, FasIII, 1B1, DAPI
H
I
J

Chapter 3

The role of Chinmo, the sex determination pathway and the microRNA *let-7* in sex maintenance in the adult Drosophila ovary and testis

This chapter is a modified version of the manuscript in preparation, "Ma Q, de Cuevas M, Matunis E. The role of Chinmo, the sex determination pathway and the microRNA *let-7* in sex maintenance in the adult Drosophila ovary and testis"

Summary

Sexual identity is continuously maintained in specific differentiated cell types long after sex determination occurs during development. In the adult *Drosophila* testis, the putative transcription factor Chronologically inappropriate morphogenesis (Chinmo) acts with the canonical male sex determination factor DoublesexM (Dsx^M) to maintain the male identity of somatic cyst stem cells (CySCs) and their progeny. Here we find that ectopic *chinmo* is sufficient to maintain male somatic identity in ovaries through a Dsx^M-independent mechanism. Similarly, the female sex determination factors may feminize male somatic cells in parallel with Chinmo. Finally, while ectopic expression of the miRNA *let-7*, which targets Chinmo in the Drosophila brain, downregulates Chinmo in testes strongly enough to feminize the testis, *let-7* is not required in the ovary. Our work shows that the transcriptional repressor Chinmo is necessary and sufficient to promote a male identity in the somatic cells within gonads of both sexes, extending sex maintenance to both the ovary and the testis in Drosophila.

Introduction

The phenotypic differences between males and females, or sexual dimorphism, arise from a variety of mechanisms, either genetic or environmental, across animal species. Recently it was discovered that the cell fate decisions that drive male versus female fate that are established during development are actively maintained, and that cellular transdiferentiation in vivo can occur within the adult gonad upon the loss of a single transcriptional regulator. In mice, terminally differentiated somatic cells within the adult ovary and testis have considerable plasticity, and the sexual identity of these cells, and subsequently these tissues, is actively maintained throughout adulthood. For example, loss of a transcription factor related to the insect sex-determination gene *doublesex* called Doublesex and mab3 related transcription factor 1(Dmrt1) in the testis causes the differentiated somatic cells in the adult testis (Sertoli cells) to trans-differentiate to their female counterparts (granulosa cells) (Uhlenhaut, Jakob et al. 2009, Matson, Murphy et al. 2011). Similarly, loss of the female regulator Foxl2 in the granulosa cells of the adult ovary triggers their conversion to Sertoli cells (Uhlenhaut, Jakob et al. 2009, Matson, Murphy et al. 2011). Recently it was shown that ectopic expression of Dmrt1 alone in the ovary is sufficient to silence Foxl2 expression and cause masculinization of the ovary (Lindeman, 2014).

We recently found that sex maintenance extends to Drosophila: the adult somatic stem cells in the Drosophila testis can convert to ovarian somatic stem cells and produce corresponding female progeny when the transcription factor Chronologically inappropriate morphogenesis (Chinmo) is removed from adult testes (Ma, Wawersik et al. 2014). Chinmo acts with the canonical sex determinant *doublesex*^{M} (*Dsx*^{M}) to maintain the male identity of

testis somatic cells. But it is not known whether Chinmo and Dsx^M are sufficient to promote the male fate of somatic cells in the ovary.

The Drosophila ovary and testis stem cell niches are very well defined. In the adult testis, the Janus kinase-Signal Transducer and Activator of Transcription (Jak-STAT) pathway maintains both sperm producing stem cells called germline stem cells (GSCs) and somatic stem cells called cyst stem cells (CySCs) (Fig. 3.1A). Two CySCs wrap around each GSC and are essential for GSC self-renewal and differentiation. Both types of stem cells adhere to a niche composed of quiescent somatic hub cells. Chinmo, as a Jak-STAT pathway effector, is required for CySC self-renewal (Flaherty, Salis et al. 2010). In the adult ovary (Fig. 3.1B), the stem cell niche is comprised of somatic cap cells supporting the self-renewal of 2-3 GSCs, which produce differentiating female germ cells. Somatic escort cells surround the GSCs. Two follicle stem cells (FSCs) are positioned posteriorly and opposite one another near the middle of the germarium, producing follicle precursor cells. Follicle precursor cells differentiate into somatic follicle cells and stalk cells. Follicle cells form a columnar epithelium to envelop each 16-cell cyst and form an egg chamber. Egg chambers are linked by chains of stalk cells. The morphology and behavior of somatic cells in the ovary and testis are quite distinct: the male CySCs produce squamous progeny cyst cells, which are quiescent, while female FSCs produce columnar epithelial cells which can proliferate. Jak-Stat signaling is also required in the somatic cap cells for ovarian GSC maintenance, and ectopic expression of the Jak-Stat ligand Upd is sufficient to promote GSC and escort cell proliferation (Decotto and Spradling 2005). Although the Jak-STAT signaling pathway is active in both the ovarian and testis niches, it is not clear if Chinmo has any roles in the ovary, or whether it is sufficient to promote male identity of the ovarian stem cells, as suggested by its requirement in maintaining

the male identity of adult CySCs. Since Chinmo works though the canonical male determinant DsxM to regulate the sex maintenance of adult male CySCs in the testis, but the role of the canonical sex determination pathway members in adult testes and ovaries is poorly understood, we also examined the requirements for these factors in sex maintenance of somatic cells in adult Drosophila gonads. We found that Chinmo is sufficient to promote the male identity of ovarian follicle stem cells, which is consistent with its function in testes. Furthermore, sex determinants are also required for sex maintenance in testes and ovaries, but the ovarian phenotypes generated by overexpressing *chinmo* or the male sex determiant Dsx^{M} are not the same. This is consistent with our finding that Chinmo has Dsx^{M} independent targets in maintaining CySC male fate. Thus, canonical sex determination factors and Chinmo are both necessary and sufficient for sex maintenance, but they might function through overlapping but different mechanisms.

Results

Chinmo is not required in germline or somatic cells in the Drosophila ovary

Our prior finding that *chinmo* maintains the male identity of somatic cells in the adult Drosophila testis prompted us to ask if it has any roles in the adult ovary. Our prior work suggested that *chinmo* may not be expressed or required in adult ovarian somatic cells. Consistent with this hypothesis, Chinmo transcripts are low to undetectable in wild-type ovaries (Gan, Chepelev et al. 2010), and Chinmo is undetectable in the somatic cells of ovaries immunostained with anti-Chinmo antisera (Fig. S3.1B). Since *chinmo* is essential during development (Zhu, Lin et al. 2006, Flaherty, Salis et al. 2010), we used the temperature-inducible $Gal80^{TS}$ system to conditionally knock down *chinmo* in adult females, thereby circumventing embryonic lethality. Since the cells within the adult testis that undergo sex transformation upon loss of Chinmo are the somatic stem cells, we were particularly interested in examining the requirements, if any, for *chinmo* in the somatic stem cells within the germaria, which are the follicle stem cells (Fig. 3.1B). We began by reducing *chinmo* by conditionally expressing two independent RNAi lines against *chinmo* in the ovarian soma of the germarium. Tools to manipulate gene expression specifically in follicle stem cells do not exist, but there are Gal4 drivers that are expressed in small subsets of somatic cells in the germarium. One is the c587-Gal4 driver, which is expressed specifically in escort cells, follicle stem cells and early follicle cells (Eliazer, Shalaby et al. 2011) (Fig. S3.1G). After conditional knockdown of *chinmo* for 1-3 weeks in adult flies, we dissected, fixed and immunostained ovaries and looked for any morphological differences between our experimental samples and wild-type controls. They were indistinguishable (Fig. S3.1D). Similar results were obtained after repeating this *chinmo* knockdown with the somatic driver

eyaA3-Gal4, which is expressed in a pattern that overlaps that of c587 in the germaria but also extends to follicle cells and stalk cells (Fig. S3.1E). We also tested the requirement for *chinmo* in the the adult female germline but this did not yield any obvious phenotypes (Fig. S3.1F). Consistent with these results, ovaries from adult females bearing the partial loss-of-function allele of *chinmo* denoted $chinmo^{Sex\ Transformation}$ ($chinmo^{ST}$) was also phenotypically indistinguishable from wild-type ovaries (data not shown). Together, these data indicate that little or no Chinmo protein is detectable in the adult ovary, and that *chinmo* does not have a major role within adult ovarian germline or somatic stem cells.

Forced expression of Chinmo in the adult ovarian soma disrupts oogenesis

Since Chinmo promotes the male identity of somatic stem cells in the adult testis, but has no apparent role in the ovary, we next asked whether driving ectopic Chinmo expression is sufficient to promote the male identity of somatic cells in the adult ovary. We again combined the somatic driver c587-Gal4, which is expressed in escort cells, follicle stem cells and early follicle cells, with $Gal80^{TS}$ to achieve conditional expression of ectopic Chinmo in adult somatic cells. We confirmed the efficacy of ectopic *chinmo* expression by immunostaining ovaries with anti-Chinmo antisera, which revealed a strong up-regulation of Chinmo expression specifically within cells expressing the c587-Gal4 driver (Fig. 3.3A). Prior to induction of ectopic *chinmo* expression, somatic *chinmo*-overexpressing ovaries were phenotypically indistinguishable from wild-type when examined for Vasa, which marks germ cells, FasIII, which highlights follicle cell membranes, and DAPI counterstaining, which reveals all nuclei (Fig. 3.1C-D). However, these ovaries developed a progressive phenotype upon transgene induction (Fig. 3.1E-J). As expected, based on the pattern of the c587 Gal4 driver, the phenotype initiated specifically within the germaria. In the earliest manifestation of

the phenotype (at four days post-induction), germaria accumulated Vasa-positive germline cysts with cystocytes containing branching fusomes; these germ cells were associated with Vasa-negative somatic cells (Fig. 3.1E). The nuclei of these somatic cells were intermingled with the germ cells (Fig. 3.1G), and occupied positions distinct from that of escort cells, which typically have peripherally located nuclei (Fig. 3.1B). In contrast, the germline stem cells (arrowhead) and their supporting niche cells (cap and terminal filament cells) appeared unaffected (Fig. 3.1F), as did cells that had exited the germarium (Fig. 3.1E, arrow), which is expected since c587 driver is not expressed in cap and terminal filament cells (Eliazer, Shalaby et al. 2011). By eight days of induction, the phenotype was more severe. Distended germaria were filled with early germ cells and clusters of somatic cells expressing high levels of the adhesion molecule Fasciclin III (Fig. 3.1H, arrowhead). These somatic cells did not envelop the germline but instead accumulated at the periphery of the posterior end of the germaria. Differentiating germ cell cysts at later stages of oogenesis, which morphologically resembled nurse cells, also appeared in these germaria. The accumulation of the somatic cells and differentiating germ cells in the germaria suggests that the germ cells cannot be packaged into normal egg chambers because the normal function of the follicle stem cell progeny (the follicle cells) is disrupted (Fig. 3.1H). As the duration of ectopic *chinmo* induction increased, differentiating germ cells died and the germaria filled with germ cells which, based on their fusome morphology, are undifferentiated cells (Fig. 3.1I, J). These undifferentiated germ cells are surrounded by many overproliferating intermingling somatic cells. The posteriorly located cluster of Fasciclin III-positive somatic cells remained, but egg chambers were no longer detected outside the germarium. This progressive phenotype suggests that ectopic expression of *chinmo* in the somatic cells of the germaria interferes with normal somatic cell

differentiation, and that germ cell differentiation is blocked as a secondary consequence. We obtained a very similar phenotype after conditionally ovexpressing *chinmo* under the control of a second somatic driver (Traffic Jam Gal4, Fig. S3.1I, Fig. S3.2B-C). Since similar phenotypes are also observed when the female germ cells reside in a male somatic environment during development (Hempel, Kalamegham et al. 2008), these data support the hypothesis that Chinmo may be sufficient to masculinize the adult ovarian somatic cells.

Ectopic expression of Chinmo in the adult ovarian soma is sufficient to promote the expression of male-specific markers

Since ovaries with ectopic Chinmo in the ovarian soma have a phenotype consistent with a female-to-male somatic sex transformation, we next examined male-specific markers to ask if these ovaries are becoming masculinized. Very few such markers exist. We identified an enhancer trap inserted in the *escargot* gene, referred to here as esg-GFP, that is expressed in the early germ cells, CySCs and their early progeny in the testis, but is undetectable in the ovary. Introducing esg-GFP into flies conditionally overexpressing *chinmo* in the adult ovarian soma revealed that, as expected, this enhancer trap was undetectable in the ovary before induction of *chinmo* expression (Fig. 3.2A-B). In contrast, after three days of ectopic *chinmo* expression, 10-20% of ovarioles developed the mild phenotype described in Fig. 3.1E, and esg-GFP became apparent in a subset of somatic cells in these ovarioles, specifically within the germaria. Based on their morphology and position, these somatic cells are likely to be follicle stem cells and/or their earliest progeny. Interestingly, at the earliest stages of induction, in the subset of ovarioles which contain three or fewer esg-positive cells, these cells were positioned at the periphery of the germaria in the positions where follicle stem cells

typically reside. However, markers that molecularly distinguish follicle stem cells from their immediate daughters have not been identified. At this early time point, esg-GFP was undetectable in all other somatic cells in the germaria including terminal filament cells, cap cells and escort cells, although the c587 driver is strongly expressed in both cell types (Fig. 3.2C). This suggests that follicle stem cells and/or their earliest progeny, but not escort cells, are the cells that become sex transformed upon ectopic *chinmo* expression in the ovary. As ectopic *chinmo* was expressed for longer periods of time, many more esg-GFP-positive somatic cells accumulated in the germaria, but esg-GFP remained undetectable in the cells at the anterior tip of the germarium (Fig. 3.2E-F). We confirmed these results by substituting the somatic Traffic Jam-Gal4 driver for c587-Gal4, which yielded a very similar phenotype (Fig. S3.2A-C). We also repeated these experiments using a second enhancer trap inserted in *escargot* (escargot-lacZ, or M5-4). M5-4 is a sexually dimorphic marker that has been used intensively in identifying male fate of the germ cells (Wawersik, Milutinovich et al. 2005, Sheng, Posenau et al. 2009), and marks early germ cells and hub cells in adult wild-type testes (Fig. S3.2D). Importantly, M5-4 is not detected in wild-type ovaries ((Stephen 2000, Ma, Wawersik et al. 2014), Fig. 3.2E). After a short period of *chinmo* induction (5 days) M5-4 remains undetectable in the ovary (data not shown). However, this male marker appears after 20 days of ectopic *chinmo* induction. In contrast to esg-GFP, which is detectable early on in the follicle stem cells and/or their progeny, M5-4 marks a few undifferentiated germ cells in the ovary following *chinmo* overexpression (Fig. 3.2F). This further supports our hypothesis that Chinmo is sufficient to masculinize the ovary, and also suggests that masculinization of the ovarian soma may eventually alter the identity of neighboring germ cells. This is consistent with previous work showing that genetically female (XX) germ cells can generate

cells resembling spermatogonia when they reside in a masculinized somatic environment during development (Hempel, Kalamegham et al. 2008). Consistent with the hypothesis that expression of M5-4 in the germline is an indirect effect of *chinmo* expression in the soma, rather than an artifact of leaky expression of *chinmo* in the germline, driving *chinmo* expression directly in ovarian germ cells does not produce any obvious phenotypes (Fig. S3.1C). The germ cells adjacent to the Chinmo-overexpressing somatic cells are overproliferating and a few are able to express a male specific marker, suggesting that the masculinized somatic cells can communicate with germ cells to block their differentiation and induce male fate. This is also consistent with the role of Jak-Stat signaling during in sex determination of somatic gonadal cells during development, which subsequently promotes the male identity of germ cells in the embryonic testis (Jinks 2000, Wawersik, Milutinovich et al. 2005). Together, our data indicate that induction of ectopic *chinmo* expression in the somatic cells is sufficient to masculinize adult ovarian somatic cells and some germ cells. Our data also suggest that female somatic stem cells (follicle stem cells) and/or their early progeny, but not neighboring escort cells, are the first cells to acquire male identity under these conditions.

Chinmo and the canonical sex determinant Dsx^M masculinize the ovary independently

Our previous data showed that *chinmo* acts with the canonical sex determination pathway member Dsx^M to promote male identity maintenance of CySCs in the adult testis [Ma, 2014]. However, loss of *chinmo* and loss of *dsx* yield overlapping but distinct phenotypes, indicating that *chinmo* has targets in addition to *dsx* in sex maintenance, and vice versa [Ma, 2014]. Therefore, we thought it likely that ectopic expression of Dsx^M in the ovarian soma could partially phenocopy ectopic expression of *chinmo* in these cells. We used the same strategy

described above to conditionally express Dsx^M in the somatic cells of adult germaria (using c587-Gal4 and $Gal80^{TS}$). This yielded ovaries containing a mixture of degenerating ovarioles and germaria filled with overproliferating early, undifferentiated germ cells. Some of these germ cells of mixed stages were fused within egg chambers (Fig. 3.3D-F, Fig. S3.2B-F). We confirmed these results by substituting the somatic Traffic Jam-Gal4 driver for c587-Gal4, which yielded a very similar phenotype (Fig. S3.3B). This suggests that overexpressing Dsx^M might masculinize the somatic cells in the germaria since these phenotypes are also observed when female germ cells are in a male somatic environment during development (Hempel, Kalamegham et al. 2008). We hypothesize that ectopic Dsx^M specifically disrupts the maintenance of adult ovarian morphology by interfering with sex identity rather than cell viability, since ectopic Dsx^M expression alone is not sufficient to yield any obvious testis phenotypes (Fig. S3.2C). We conclude that ectopic expression of Dsx^M and *chinmo* in the adult ovary produce distinct but overlapping phenotypes, which suggests that *chinmo* is not acting solely through Dsx^M to masculinize the ovary. Consistent with this hypothesis, *chinmo* overexpression in the adult ovary is not sufficient to cause the expression of the male sex determinant Dsx^M (Fig. 3.3B-C). Since *chinmo* is required for Dsx^M expression in the adult testis, but the Dsx^M signal can be difficult to detect, we verified that our assay was sensitive enough to detect Dsx^M in ovaries by immunostaining ovaries ectopically expressing Dsx^M in the adult ovarian soma for this antigen (Fig. S3.3A). Together, these data support the hypothesis that ectopic *chinmo* promotes male somatic identity in ovaries through a Dsx^M-independent mechanism.

The female sex determination factors may feminize male somatic cells in parallel with Chinmo

Since ectopic expression of the canonical male sex determination factor Dsx^{M} causes defects in the ovary that are consistent with a somatic sex transformation, we next wondered if the ectopic expression of female sex determinants might also be sufficient to masculinize the adult testis. Our previous work showed that the canonical female sex determinants Sex lethal (Sxl), the female form of Transformer (Tra^{F}) and the female form of Doublesex (Dsx^{F}) are not ectopically expressed in testes lacking *chinmo*, but it remained possible that these factors could phenocopy loss of *chinmo* when they are expressed ectopically on their own. Therefore, we again conditionally expressed Sxl, Tra^{F} or Dsx^{F} specifically in the CySC lineage of adult testes (using c587-Gal4 and $Gal80^{TS}$). Before transgene induction, testes were phenotypically indistinguishable from wild-type testes (Fig. 3.4A, D, G). After overexpression of the female determinants Sxl, Tra^{F} or Dsx^{F} in the adult CySC lineage, testes of all three genotypes initially acquired small aggregates of somatic cells and overproliferating, undifferentiating germ cells (Fig. 3.4A-I), which is very similar to the phenotype we observed in testes lacking *dsx* (Ma, 2014). The somatic aggregates are consistent with phenotypes that arise when somatic cells lose their male identity and gain a female identity, and the overproliferating germ cells likely represent a mismatch between the sex identity of the germline and the soma, as we previously can occur in young $chinmo^{ST}$ mutant testes (Ma, 2014). However, testes expressing ectopic female determinants only partially phenocopy the $chinmo^{ST}$ mutant phenotype. The earliest morphological aspect of this phenotype is the formation of somatic cell aggregates in the testis apex, which is followed by the appearance of the progeny of sex transformed CySCs: a full layer of follicle-like cells lines the testis periphery. In testes expressing ectopic female

determinants clusters of somatic cells develop, but follicle-like cells do not. In support of the hypothesis that these phenotypes reflect defects in female fate maintenance, rather than non-specific effects of high level expression of these factors, driving ectopic Sxl, Tra^F or Dsx^F in the adult ovarian soma (using c587-Gal4) produces no obvious phenotypes (Fig. S3.4A-C). Together, these data indicate that the ectopic expression of canonical female sex determinants may be sufficient to promote the feminization of adult CySC lineage cells, but they may act in a manner that is distinct from ectopic *chinmo* expression.

Although Sxl, Tra^F and Dsx^F are each able to produce phenotypes in the testis that are consistent with a sex transformation of somatic cells, Sxl is also required for female germ cell sex determination and maintenance (Hashiyama, Hayashi et al. 2011, Shapiro-Kulnane, Smolko et al. 2015). Therefore, we asked if conditionally overexpressing Sxl in adult male germ cells (using *nanos*-Gal4) is sufficient to promote the female fate of germ cells. We found that ectopic Sxl in germ cells in testes is sufficient to cause adult male germ cell loss (Fig. S3.4D). Since female germ cells also die when they reside in male soma during development (Hempel, Kalamegham et al. 2008), we consider it likely that the loss of adult male germ cells upon expression of Sxl in the germ cells is due to the feminization of the germ cells. Furthermore, this effect is specific to Sxl overexpression, since ectopically expressing Tra^F or Dsx^F in adult male germ cells in the testis does not yield any obvious phenotypes (Fig. S3.4F), as expected, since Sxl does not act through Tra or dsx in the germ cells during germ cell sex determination in development (Whitworth, Jimenez et al. 2012). Together these data indicate that the ectopic expression of canonical female determinants in the adult testis is sufficient to disrupt the male fate and promote the female fate of adult CySC lineage cells, but the

canonical sex determination machinery is not sufficient to fully convert cells in the adult CySC lineage to follicle stem cell-like cells as does *chinmo*.

Since ectopic expression of the female sex determination pathway members in the adult testis is sufficient to feminize CySC lineage cells, we next asked if these female factors genetically interact with *chinmo* during the process. We used c587-Gal4 to conditionally overexpress Dsx^F together with *chinmo* RNAi-knockdown in the CySC lineage of adult testes. We found that this dramatically enhances the feminization phenotype (Table 3.1). After 4 days of transgene induction, only 6.3% of Chinmo-RNAi testes (n=64) and 0% of Dsx^F overexpressing testes (n=42) have the earliest aspect of the $chinmo^{ST}$ testis phenotype (the appearance of somatic cell aggregates). In contrast, 98.4% of the testes have this phenotype when *chinmo* knockdown and and Dsx^F overexpression are combined. We conclude that reducing *chinmo* while overexpressing Dsx^F greatly accelerates the feminization of the adult CySC lineage cells. Thus, *chinmo* and Dsx^F interact genetically, and both *chinmo* (Ma, Wawersik et al. 2014) and the canonical sex determination pathway members are required to maintain the adult somatic sex identity of cells in the testis.

Ectopic expression of miRNA *let-7* in the testis soma is sufficient to down-regulate Chinmo and cause the sex conversion phenotype

Chinmo protein is highly expressed in the testes and is required to continually maintain the male identity of adult CySCs (Ma, Wawersik et al. 2014), but this protein is undetectable in the ovary (Fig. S3.1B). Since *chinmo* transcripts are directly regulated by microRNAs co-transcribed from the *let-7-Complex* (*let-7-C*) in the developing Drosophila brain, and ectopic *let-7-C* is sufficient to downregulate Chinmo protein levels in mushroom body neurons (Wu,

2012), we asked whether ectopic *let-7-C* expression in somatic cells of the adult testis would phenocopy loss of *chinmo*. We conditionally overexpressed *let-7-C* in the adult CySC lineage (using c587-Gal4 and $Gal80^{TS}$) and immunostained testes with anti-Chinmo antisera. Before *let-7-C* overexpression, Chinmo was expressed as expected in hub cells, CySC lineage cells, and at lower levels in germ cells (Fig. 3.5A); these testes appeared phenotypically normal (Fig. 3.5C). After 5 days of *let-7-C* transgene induction, Chinmo protein levels decreased in the CySCs and cyst cells but remained expressed in the hub and germ cells (Fig. 3.5B), which was expected given that we did not express the transgene in the latter two cell types. After extending the period of ectopic *let-7-C* expression for several more days, testes acquired follicle-like cells, strikingly phenocopying the *chinmo* somatic sex transformation phenotype (Fig. 3.5D). Immunostaining for Chinmo at this later timepoint indicated that the somatic aggregates and the follicle-like cells lacked Chinmo, while the hub cells and germ cells still expressed Chinmo (Fig. S3.5D-E). We confirmed this result using two additional CySC lineage Gal4 drivers (eyaA3-Gal4, tj-Gal4) which gave very similar results (Fig. S3.5A-B). We also obtained the same phenotype by overexpression of a single *let-7-complex* miRNA (miRNA *let-7*), further confirming this result (Fig. 3.5D). Finally, as expected, since *chinmo* is not required in the germ cells in the testes, overexpressing *let-7* in the germ cells yielded no obvious phenotypes (Fig. S3.5C). Together these data indicate that ectopic *let-7* is sufficient to down-regulate Chinmo in the adult CySC lineage, and the resulting decrease in Chinmo levels is severe enough to feminize the testis.

let-7 might not repress Chinmo in the ovary

Since ectopic *let-7* is sufficient to down-regulate Chinmo in the testis and cause the testis sex conversion phenotype, we hypothesized that *let-7* may be required to maintain Chinmo at undetectable levels in the wild-type ovary, thereby having an important role in preventing the female to male conversion of somatic cells. However, it is also possible that the regulation of Chinmo occurs at the transcriptional rather than the post-transcriptional level. Therefore, we examined ovaries from adult female flies carrying multiple different *let-7-C* deletions. We did not find any obvious ovary phenotypes (Fig 3.6A-B). Consistent with this observation, we also did not detect any ectopically expressed Chinmo in these ovaries by immunostaining (Fig. 3.6D-E). We conclude that *let-7* is not required to repress *chinmo* expression in the adult ovary.

Discussion

The masculinization phenotype we observe in Drosophila ovaries in response to ectopic Chinmo expression in the somatic cells of adult females suggests that Chinmo is sufficient to promote the male identity of ovarian somatic cells. This is consistent with Chinmo's role in maintaining the male identity of CySCs in the adult testis. Thus, the sexual identity of the somatic gonadal cells in either the ovary or the testis can be changed by the manipulation of single genes in organisms ranging from files to mammals (Uhlenhaut, Jakob et al. 2009, Matson, Murphy et al. 2011). Since Chinmo is necessary and sufficient to promote male identity in the testis and ovary, it is possible that Chinmo homologues could function more broadly in sex maintenance in other organisms.

Given that the male specific marker esg-GFP is initially detected in the posterior half of germaria, which contains FSCs and their early progeny, and that it initially appears in cells positioned were FSCs and/or their immediate progeny reside, we think that FSCs are most likely the cells that initially undergo a female-to-male sex conversion upon ectopic *chinmo* expression. With increasing periods of *chinmo* overexpression, somatic cells with esg-GFP accumulate in the ovary, where they intermingle with overproliferating undifferentiated germ cells, which resemble the germ cells that accumulate when the female determinant Sxl is removed from whole ovaries by *sxl* or *snf* mutations (Decotto and Spradling 2005, Shapiro-Kulnane, Smolko et al. 2015). However, to unambiguously distinguish FSCs from their immediate progeny requires lineage tracing. In the future, combining lineage tracing of FSCs with genetic tools for Chinmo overexpression could be informative. We suspect that the sex-transformed cells divide often and give rise to progeny that continue to express esg-GFP, since we rarely see ovarioles that contain only a few esg-GFP-positive cells. However, it remains

possible that cells in the germaria other than follicle stem cells and their early progeny are the source of sex-transformed cells. Again, lineage tracing should be informative.

In mice, the sex-determining region of chromosome Y (*SRY*) gene in the fetal gonad triggers male sex determination by activating the male determinant SOX9 (Matson and Zarkower 2012). Although *Dmrt1* is required for maintaining the male identity of Sertoli cells in postnatal mice, it is not required for the initial sex determination (Matson and Zarkower 2012). However, recent work showed that transgenic overexpression of *Dmrt1* in female somatic gonadal cells causes trans-differentiation of adult somatic granulosa cells to Sertoli cells, which indicates that Dmrt1 is sufficient to convert fully differentiated adult female somatic cells into functional male somatic cells. Interestingly, this somatic sex conversion is independent of activation of the male sex determinant SOX9 (Lindeman, Gearhart et al. 2015, Zhao, Svingen et al. 2015). Our data showed that overexpressing *dsx* in ovarian soma in adult Drosophila can give rise to phenotypes which suggest a disruption in sex identity of somatic cells. The phenotype caused by *chinmo* overexpression is also independent of *dsx* since the phenotypes are quite distinct and Dsx^{M} is not ectopically expressed in germaria with Chinmo overexpression. This is consistent with the idea that Chinmo is promoting *dsx* expression at the transcription level in the CySC lineage in the testis (Ma, Wawersik et al. 2014). In this case, even if *chinmo* overexpression is sufficient to promote *dsx* transcription in the ovary, presence of the female sex determinants, which we show are needed in the adult ovary for sex maintenance, will provide the splicing cascade that converts *dsx* transcripts into the female form ($Dsx^{F)}$ rather than the male determinant Dsx^{M}. Together, these data support the hypothesis that Chinmo masculinizes the ovary in a Dsx^{M} independent manner.

Our data demonstrated that overexpression of the let-7 miRNA in CySC lineage cells in adult testes can cause down-regulation of Chinmo and phenocopy loss of *chinmo*. An age-related increase in levels of let-7 with age is also known to down-regulate Imp in the hub, which in turn mediates a slight age-related decrease in GSC number (Toledano, D'Alterio et al. 2012). This finding is consistent with the general view that miRNAs normally work by coordinately fine-tuning the expression of many targets (Ebert and Sharp 2012). Perhaps manipulating let-7 causes different phenotypes in different cells due to distinct functions of targets in different cells. Since ectopically expressed let-7 is sufficient to down-regulate Chinmo in testes to levels that produce a phenotype that is indistinguishable from a very strong loss-of function allele, this suggests that let-7-C might normally downregulate Chinmo levels in the ovary to prevent masculinization of the somatic cells. However, we did not detect ectopic Chinmo expression or any obvious ovarian phenotypes similar to *chinmo* overexpression when knocking out let-7, even in whole flies that were displaying the neurological phenotypes characteristic of let-7-C deficiency (Sokol, Xu et al. 2008). In a previously published study, knocking out let-7 in the ovary was shown to give a mild phenotype, in which the number of spectrosome-containing early germ cells increases from around 4 to 7 (Konig and Shcherbata 2015). However, the authors of that study also did not report any phenotype similar to *chinmo* overexpression. It is possible that *chinmo* might not be transcribed, or there might be some other factors, which can keep Chinmo off.

Our finding that somatic stem cells/somatic cells undergo sexual transformation may provide unique insight into how sex maintenance is regulated at a cellular and molecular level more generally. Sex transformation in adult *Drosophila* testes provides a highly tractable genetic system to study cellular mechanisms of how somatic sexual identity is actively

maintained by different genes in *dsx*-dependent/independent ways. This may provide insight into human testicular cancers, such as granulosa cell tumors, that may be linked to altered somatic sexual identity (Hanson and Ambaye 2011). Analysis of Chinmo and Dsx/Dmrt1-mediated sex maintenance pathways in somatic stem cells may also yield insight into the maintenance of sexual dimorphism in other organs, such as the mammalian liver, and provides a unique model to study adult stem cell transdifferentiation *in vivo*.

Experimental Procedures

Fly stocks and cultures

Fly stocks were raised at 25 ˚C on standard molasses/yeast medium unless otherwise indicated. The following fly stocks were used: *UAS-FL-chinmo* and *UAS-5'UTR-chinmo* (Zhu et al., 2006), *eyaA3-Gal4* (Leatherman and DiNardo, 2008), *M5-4* (Gönczy and DiNardo, 1996), *esg-GFP* (CB02017, from Spradling lab), *c587-Gal4* (Kai and Spradling, 2003), *nanos-Gal4-VP16* (Van Doren et al., 1998), *tj-Gal4* (Drosophila Genetic Resource Center), *UAS-Sxl* (from Horabin lab), *UAS-dsx^{F}* (from Baker lab), *UAS-dsx^{M}* (Lee et al., 2002), *let-7-C^{GKI}/CyO* and *let-7-C^{KO1}/CyO* (lack the whole let-7-C locus), *UAS-let-7-C*, and *UAS-let-7* (Sokol, 2008). *y w* flies were used as control flies. Other fly stocks were from VDRC or BDSC.

Immunostaining

Testes and ovaries were dissected, fixed, and stained as described previously (Matunis 1997). Tyramide signal amplification (Invitrogen) was used to increase sensitivity of rat anti-Dsx^{M} (from B. Oliver, 1:500 dilution). The following antibodies were also used: rabbit anti-Vasa (d-260) and goat anti-Vasa (dN-13) (Santa Cruz Biotechnology, 1:400); rabbit anti-GFP (Torrey Pines Biolabs, 1:10,000); chicken anti-GFP (Abcam, 1:10,000); mouse anti-β-Galactosidase (Promega, 1:1000); mouse anti-1B1 (1:25), mouse anti-Fasciclin III (1:50), and mouse anti-Armadillo (1:50), all from Developmental Studies Hybridoma Bank, University of Iowa; rat-anti-Chinmo (from N. Sokol, 1:500); rabbit anti-ZFH1 (from R. Lehmann, 1:5000); and guinea pig anti-Tj (from D. Godt, 1:4000. Alexa fluor-conjugated secondary IgG (H+L) antibodies were diluted at 1:200 for 568 and 633 conjugates and 1:400 for 488 conjugates. Secondary antisera were: goat anti-rat 488, goat anti-rabbit 488 and 568, goat anti-mouse 488

and 568, goat anti-chick 488, and goat anti guinea-pig 568 (Molecular Probes/Invitrogen). DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1 mg/ml.

Conditional gene expression

To overexpress or knock down genes in a cell specific and temporal manner, cell type specific Gal4 drivers were used in combination with a temperature-sensitive allele of the Gal4 repressor ($Gal80^{[ts]}$) to conditionally express transgenic RNAi or overexpression constructs of different genes. In order to induce transgene expression only in adult flies but not during development, flies were grown at the permissive temperature of 18 °C in which Gal4 expression is repressed, and shifted to the restrictive temperature of 29 °C or 31 °C as young adult flies to induce overexpression or RNAi knock down of different genes for various lengths of time.

Microscopy and Image Analysis

Fixed testes were mounted in Vectashield (Vector Labs), imaged with a Zeiss LSM 5 Pascal or LSM 510 Meta, and analyzed using the Zeiss LSM Image Browser software; panels are single confocal sections unless stated otherwise.

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Figure Legends

Figure 3.1: Overexpressing *chinmo* in the somatic cells of adult cause defects in egg chamber formation.

(A) Illustration of a wild-type *Drosophila* testis apex (adapted from de Cuevas and Matunis, 2011). Germline stem cells (GSCs, dark yellow) and somatic cyst stem cells (CySCs, dark blue) adhere to the hub (green). GSCs, which contain spherical fusomes (red), produce differentiating male germ cells (spermatogonia, yellow), which contain branched fusomes. Approximately two somatic cyst stem cells (CySCs, dark blue) flank each GSC; CySCs produce squamous, quiescent cyst cells (light blue), which encase differentiating germ cells. **(B)** Illustration of a wild-type *Drosophila* germarium and egg chamber (adapted from Ma et al., 2014). Terminal filament cells (dark green) and cap cells (light green) support GSCs (dark yellow), which produce differentiating female germ cells (light yellow). Escort cells (grey) surround dividing germ cells in the anterior half of the germarium. Two somatic follicle stem cells (FSCs, magenta) produce follicle precursor cells (light pink), which differentiate into follicle cells (orange) and stalk cells (blue). Each egg chamber contains a cluster of 16 germ cells surrounded by a monolayer of columnar epithelial follicle cells. Egg chambers are linked by chains of stalk cells. **(C-J)** Immunofluorescence detection in adult ovaries of FasIII (green at cell periphery) to visualize somatic cell membranes and 1B1 (green in germ cells) to mark fusomes. Vasa marks germ cell (red); DAPI marks all nuclei (blue); anterior is to the left. Before induction of *chinmo* overexpression, the adult ovariole (C) and germarium (D) look normal. Escort cells (white arrows) express lower levels of FasIII and follicle cells (yellow arrow) express higher levels. GSCs (arrowhead) are attached to cap cells (open arrowhead). After *chinmo* overexpression in adult somatic cells for 4 days (E-G), defects in egg chamber

formation are apparent. The boxed areas in (E) are enlarged in (F) and (G). The stem cell niche looks normal (F), but clusters of differentiating germ cells intermingle with somatic cells (G, somatic cells outlined) accumulate at the posterior of the germarium. Based on the position and the high FasIII staining of the somatic cells they do not look like escort cells. Based on the morphology and arrangement, they do not look like follicle cells either. After additionaly days of *chinmo* overexpression (H-I), ovaries fail to form normal egg chambers, and germaria are filled with overproliferating early germ cells and somatic cells. (J) Overexpression of *chinmo-5'UTR* (without the 3'UTR) gives the same phenotype as full length *chinmo* overexpression. Scale bars = 20 μm.

Figure 3.2: Somatic cells in the germarium express a male-specific marker upon overexpresion of *chinmo*

(A-F) Immunofluorescence detection in the adult testis (A) and ovary (B-F) of GFP (green) to visualize expression of a male-specific enhancer trap *esg-GFP* and Vasa (red) to visualize germ cells. Before induction of *chinmo* overexpression, the adult testis (A) and ovary (B) look normal. In the testis, *esg-GFP* is expressed in the hub (dashed outline), GSCs (arrowheads) and early germ cells, and CySCs and early cyst cells (arrows), but it is not expressed in the ovary. After *chinmo* is overexpressed in somatic cells in adult germaria for 3 days (C), somatic cells (arrows) in the posterior of the germarium, where follicle stem cells and their early progeny reside (solid bracket), start to express *esg-GFP*. *esg-GFP* is not expressed in escort cells (arrowheads) or cap cells (open arrowhead) within the anterior half of the germarium (dashed bracket). After additional days of *chinmo* overexpressed (D-F), the ovaries develop a more severe phenotype as described in Figure 1, and *esg-GFP* is expressed in more somatic

cells including cells (F, arrows) close to the stem cell niche. DAPI marks nuclei (blue). Anterior is to the left in all panels. Scale bars = 20 μm.

Figure 3.3. Chinmo does not act through the male determinant Dsx^M to masculinize the ovary.

(A) Immunofluorescence detection of Chinmo (green). After 5 days of *chinmo* overexpression in somatic cells in the adult germarium with *c587-gal4*, Chinmo is strongly expressed in the nuclei of escort cells, follicle stem cells and early follicle cells. **(B-C)** Immunofluorescence detection of the male-specific protein Dsx^M (green). Dsx^M is not expressed in control ovaries (*c587-gal4*) (B) or in ovaries after 5 days of *chinmo* overexpression in somatic cells in the adult germarium. **(D-F)** Immunofluorescence detection in adult ovaries of FasIII (green at cell periphery) to visualize somatic cell membranes and 1B1 (green in germ cells) to mark fusomes. Before induction of Dsx^M overexpression, ovaries look normal (D). After Dsx^M is overexpressed in somatic cells in adult germaria (E-F), ovaries display a range of phenotypes including degenerating egg chambers (E, arrows) and abnormal egg chambers that are packed with early germ cells (F, arrowhead). Vasa (red) marks germ cells and DAPI (blue) marks nuclei in all panels. Scale bars = 20 μm.

Figure 3.4. Ectopic expression of female determinants in the adult testis CySC lineage disrupts testis morphology and partially phenocopies loss of *chinmo*

(A-I) Immunofluorescence detection of FasIII (green at cell periphery) and 1B1 (green in germ cell fusomes) and Vasa (red, germ cells) to visualize testis morphology before or after overexpression of different female sex determinants in the CySC lineage in adult testes.

Before overexpression of *sxl* (A), tra^F (D), or dsx^F (G), testes look normal. After *sxl* is overexpressed for 5 days (B-C), testes contain aggregates of FasIII+ somatic cells (B, arrowhead) or overproliferating early germ cells (C). After tra^F is overexpressed for 9 days (E) or 11 days (F), the testis apex contain large aggregates of FasIII+ somatic cells (E-F, arrowheads), and the germ cells overproliferate. After dsx^F is overexpressed for 7 days (H-I), germ cells fail to differentiate, and the testes acquire $FasIII^+$ somatic aggregates. Vasa (red) marks germ cells, DAPI (blue) marks nuclei, yellow arrows mark the hub, and arrowheads mark $FasIII^+$ somatic aggregates in all panels. Scale bars = 20 μm.

Figure 3.5. Overexpression of miRNA *let-7* in the adult testis phenocopies loss of *chinmo*

(A-B) Immunofluorescence detection of Chinmo (green) in testes before or after *let-7-C* overexpression in the adult CySC lineage. Before induction of *let-7-C* overexpression (A), Chinmo is expressed in CySC lineage cells (arrows), germ cells (arrowhead) and hub cells (dashed circle). After 5 days of *let-7-C* overexpression (B), Chinmo is depleted specifically in the CySC lineage (arrows) but is still expressed in germ cells (arrowhead) and hub cells (dashed circle). **(C-E)** Immunofluorescence detection of FasIII (green at cell periphery) and 1B1 (green in fusomes) to visualize the morphology of adult testes before or after overexpression of *let-7-C* or *let-7* in the adult CySC lineage. Before induction of *let-7-C* overexpression (C), the testis looks normal. After overexpression of either *let-7-C* (D, n=19/30) or *let-7* alone (E, n=16/23) in adult CySCs, testes contain monolayers of somatic follicle-like cells / monolayers of somatic cells that look like female follicle cells / and overproliferating germ cells, similar to *chinmo* mutant testes (Ma et al., 2014). Vasa (red) marks germ cells and DAPI (blue) marks nuclei in all panels. Scale bars = 20 μm.

Figure 3.6. Figure 6. *let-7* does not repress Chinmo in the ovary

(A-C) Immunofluorescence detection of FasIII (green at cell periphery) and 1B1 (green in fusomes) to visualize the morphology of adult ovaries and testes in *let-7-C* knock out flies. Both ovaries (A-B) and testes (C) look normal. **(D-F)** Immunofluorescence detection of Chinmo (green) in ovaries and testes in *let-7-C* knock out flies. Chinmo is not ectopically expressed in somatic cells in *let-7-C* knock out ovaries (D-E) but is expressed in somatic and germline cells in *let-7-C* knock out testes (F) in a pattern similar to wild-type testes (see Fig. S1A-B). Vasa (red) marks germ cells and DAPI (blue) marks nuclei in all panels. Scale bars = 20 μm.

Figure S3.1 (supports Figure 3.1). *chinmo* is not required in the ovary

(**A-B**) Immunofluorescence detection of Chinmo (green) in adult testes and ovaries. In control testes (A), Chinmo is found in the nuclei of hub cells (asterisk), CySC lineage cells (arrows), and germ cells (red) (Flaherty et al., 2010). In control ovaries (B), Chinmo is expressed weakly in a few germ cells (red) and is absent from somatic cells (arrows). (**C-F**) Immunofluorescence detection in adult ovaries of FasIII (green at cell periphery) to visualize somatic cell membranes and 1B1 (green in germ cells) to mark fusomes. Ovaries look normal when *chinmo* is overexpressed in the germ cells (C) or when *chinmo* is knocked down (via RNAi induction) in the somatic cells (D-E) or germ cells (F) in adult ovaries. (**G-I**) Immunofluorescence detection of nuclear-localized UAS-GFP (green) in adult ovaries confirms that as expected *c587-Gal4* (G) is expressed in escort cells, follicle stem cells, and some early follicle cells; *eyaA3-Gal4* (H) is expressed in some escort cells, follicle stem cells,

follicle cells, and stalk cells; and *tj-Gal4* (I) is expressed in escort cells, follicle stem cells, follicle cells, and stalk cells. Vasa (red) marks germ cells; DAPI (blue) marks nuclei; and anterior is to the left in all panels. Scale bars = 20 μm.

Figure S3.2 (supports Figure 3.2). Somatic cells and germ cells in the ovary express male-specific markers when *chinmo* is overexpressed in somatic cells

(**A-C**) Immunofluorescence detection in adult ovaries of GFP (green) to visualize expression of a male-specific enhancer trap *esg-GFP* and Vasa (red) to visualize germ cells. Before induction of *chinmo* overexpression (A), the adult ovary looks normal and does not express *esg-GFP*. After *chinmo* is overexpressed in adult somatic cells for 3 days (B), somatic cells (arrows) in the posterior of the germarium, where follicle stem cells and their early progeny reside, start to express *esg-GFP*. *esg-GFP* is not expressed in escort cells (arrowheads) or cap cells. After *chinmo* is overexpressed for additional days (C), the ovaries develop a more severe phenotype as described in Figure 1, and *esg-GFP* is expressed in more somatic cells. **(D-F)** Immunofluorescence detection of β-galactosidase (green) to visualize expression of M5-4, a male-specific *escargot* enhancer trap, and Vasa (red) to visualize germ cells. In control testes (D), M5-4 is expressed in hub cells (yellow arrow), GSCs (yellow arrowheads) and early differentiated germ cells, but not in CySC lineage cells (white arrowhead). M5-4 is absent from control ovaries (E). After *chinmo* is overexpressed in the somatic cells of the germarium for 20 days (C), M5-4 is expressed in some germ cells (arrowheads). DAPI marks nuclei (blue) and anterior is to the left in all panels. Scale bars = 20 μm.

Figure S3 (supports Figure 3). Ectopic somatic Dsx^M is sufficient to disrupt the morphology of adult ovaries but not testes

(A) Immunofluorescence detection of Dsx^M (green). After 7 days of Dsx^M overexpression in somatic cells in adult germaria, Dsx^M can be detected in some of the Dsx^M overexpressing cells (arrowheads). **(B-F)** Immunofluorescence detection of FasIII (green at cell periphery) to visualize somatic cell membranes and 1B1 (green in germ cells) to mark fusomes. After 1-2 weeks of Dsx^M overexpression in the somatic cells of adult ovaries with either *tj-Gal4* (B) or *c587-Gal4* (D-F), ovaries have a range of phenotypes including degenerating egg chambers or abnormal egg chambers containing early germ cells. After 1-2 weeks of Dsx^M overexpression in the CySC lineage in adult testes with *c587-Gal4*, the testes look normal (C). Vasa (red) marks germ cells and DAPI (blue) marks nuclei in all panels. Scale bars = 20 μm.

Figure S3.4 (support Figure 3.4). Female determinants disrupt testis morphology when overexpressed in testis somatic cells, but not in ovaries

(**A-F**) Immunofluorescence detection of FasIII (green at cell periphery), 1B1 (green in germ cell fusomes), and Vasa (red, germ cells) to visualize the morphology of adult testes and ovaries after overexpression of different female determinants. After overexpression of *sxl* (A), tra^F (B), or dsx^F (C) in the somatic cells of adult germaria, ovaries look normal. After overexpression of *sxl* in germ cells in adult testes, testes contain fewer early germ cells (D, n=15/15 testes). After overexpression of tra^F in hub cells (E, n=6/6 testes) or germ cells (F, n=19/19 testes), testes look normal. DAPI (blue) marks nuclei in all panels; yellow arrows mark the hub in panels D-F. Scale bars = 20 μm.

Figure S3.5 (supports Figure 3.5). Overexpression of miRNA *let-7* in the adult testis phenocopies loss of *chinmo*

(**A-C**) Immunofluorescence detection of FasIII (green at cell periphery) to visualize testis morphology after *let-7-C* overexpression in different types of cells in adult testes. After *let-7-C* overexpression in the adult CySC lineage with the drivers *eyaA3-gal4* (A, n=24/41) or *tj-gal4* (B, n=15/18), testes contain aggregates of somatic cells or monolayers of follicle-like cells, similar to *chinmo* mutant testes. After *let-7-C* overexpression in germ cells (C), the testes looks normal (n=31/31). Hubs are marked by yellow arrows. (**D-E**) Immunofluorescence detection of Chinmo (green) in testes after overexpression of *let-7-C* in the adult CySC lineage. Chinmo is depleted from somatic aggregates (D, arrows) and follicle-like cells (E, arrows) but remains expressed in germ cells (arrowheads) and the hub (dashed circles). D' and E' show the green channel (Chinmo) only. Vasa (red) marks germ cells and DAPI (blue) marks nuclei in all panels. Scale bars = 20 μm.

Table 3.1: Overexpressing Dsx^F enhances the Chinmo knockdown phenotype

Use c587-Gal4 to express: [1]	% testes with $FasIII^+$ somatic aggregates[2]					
	0 d	4 d	7 d	9 d	11 d	14d
UAS-chinmoRNAi-1	0 (n=31)	6.3 (n=64)	73.8 (n=103)	80.5 (n=133)	97.1 (n=35)	100 (n=59)
UAS-chinmoRNAi-1, UAS-Dsx^F	N/A	98.4 (n=63)	98.1 (n=54)	95.7 (n=47)	100 (n=35)	N/A
UAS-Dsx^F	0 (n=34)	0 (n=42)	34.1 (n=44)	47.1 (n=17)	93.8 (n=32)	N/A

[1] *UAS-chinmoRNAi-1 = chinmo^{HM04048}*

[2] All flies were raised at 18 ˚C, which suppresses RNAi induction. After eclosion, adult flies were shifted to 29 ˚C for the indicated length of time to induce RNAi

[4] For Dsx^F rescue, $p<0.0001$ at 4, 7 days, $P<0.05$ at 9 days, compared to age-matched *UAS-chinmoRNAi-1*

Figure 3.1

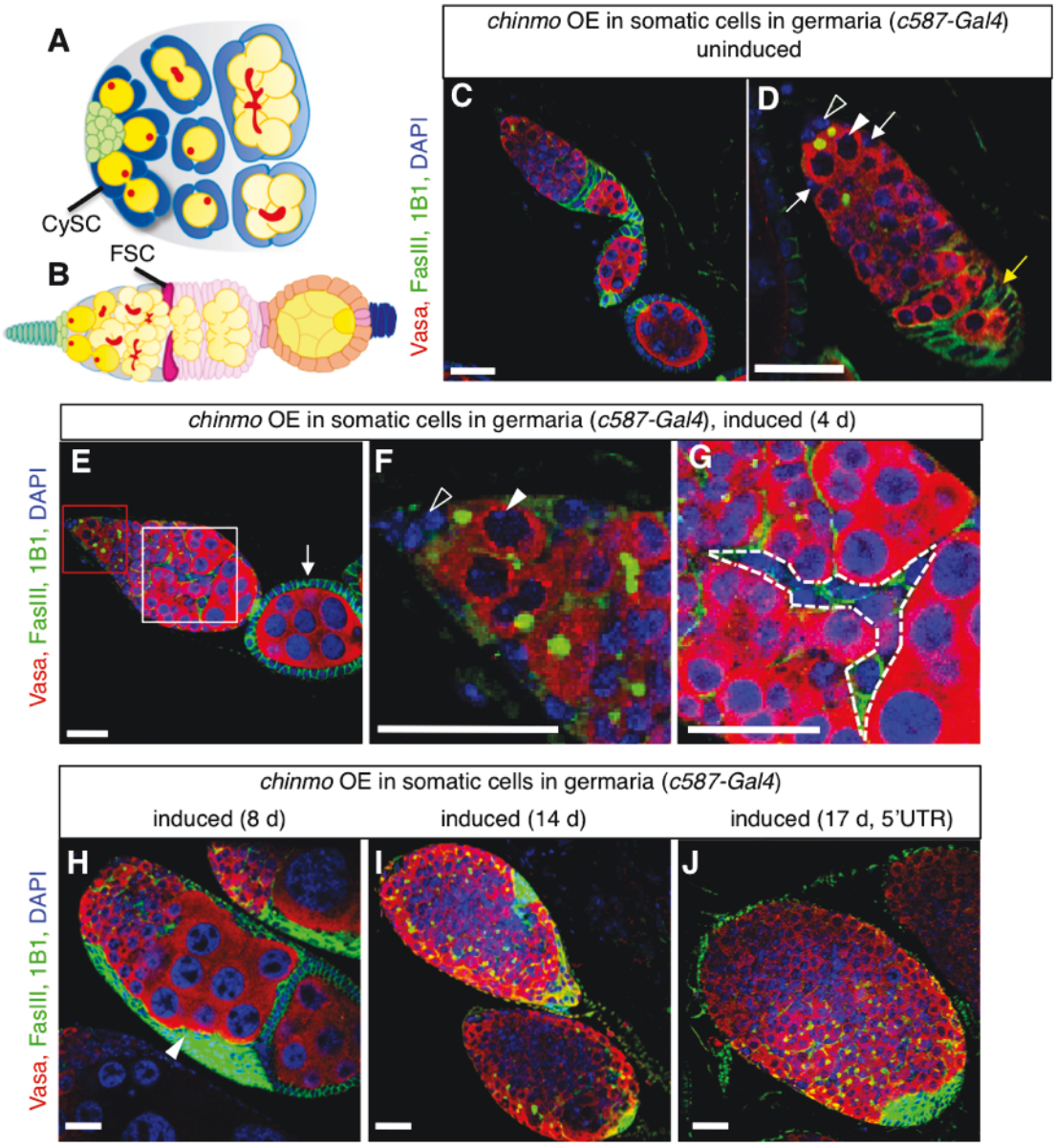

A
CySC
FSC
B
chinmo OE in somatic cells in germaria (c587-Gal4)
uninduced
C
D
Vasa, FasIII, 1B1, DAPI
chinmo OE in somatic cells in germaria (c587-Gal4), induced (4 d)
E
F
G
Vasa, FasIII, 1B1, DAPI
chinmo OE in somatic cells in germaria (c587-Gal4)
induced (8 d)
induced (14 d)
induced (17 d, 5'UTR)
H
I
J
Vasa, FasIII, 1B1, DAPI

Figure 3.2

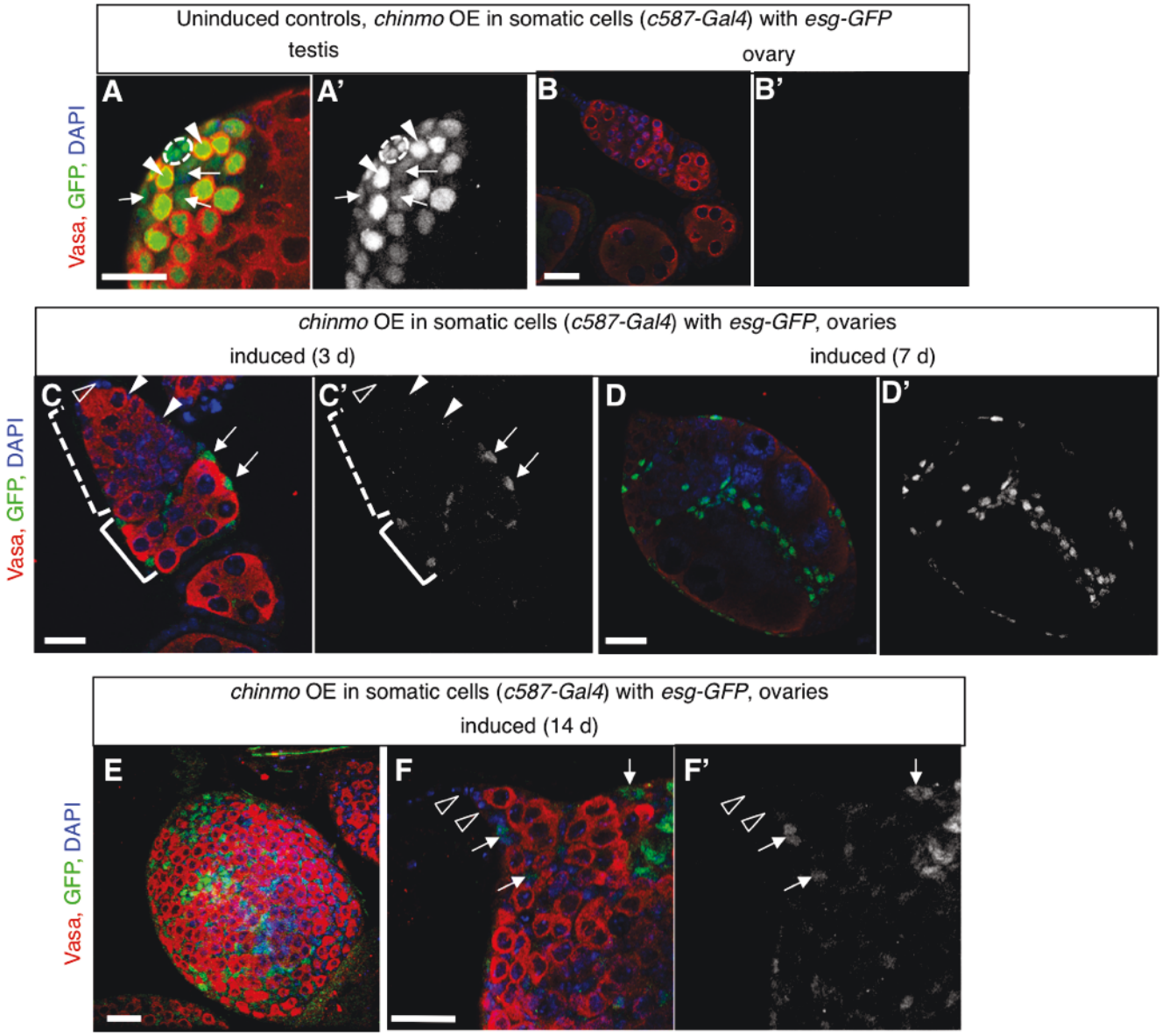

Uninduced controls, chinmo OE in somatic cells (c587-Gal4) with esg-GFP
testis
ovary
A
A'
B
B'
Vasa, GFP, DAPI
chinmo OE in somatic cells (c587-Gal4) with esg-GFP, ovaries
induced (3 d)
induced (7 d)
C
C'
D
D'
Vasa, GFP, DAPI
chinmo OE in somatic cells (c587-Gal4) with esg-GFP, ovaries
induced (14 d)
E
F
F'
Vasa, GFP, DAPI

Figure 3.3

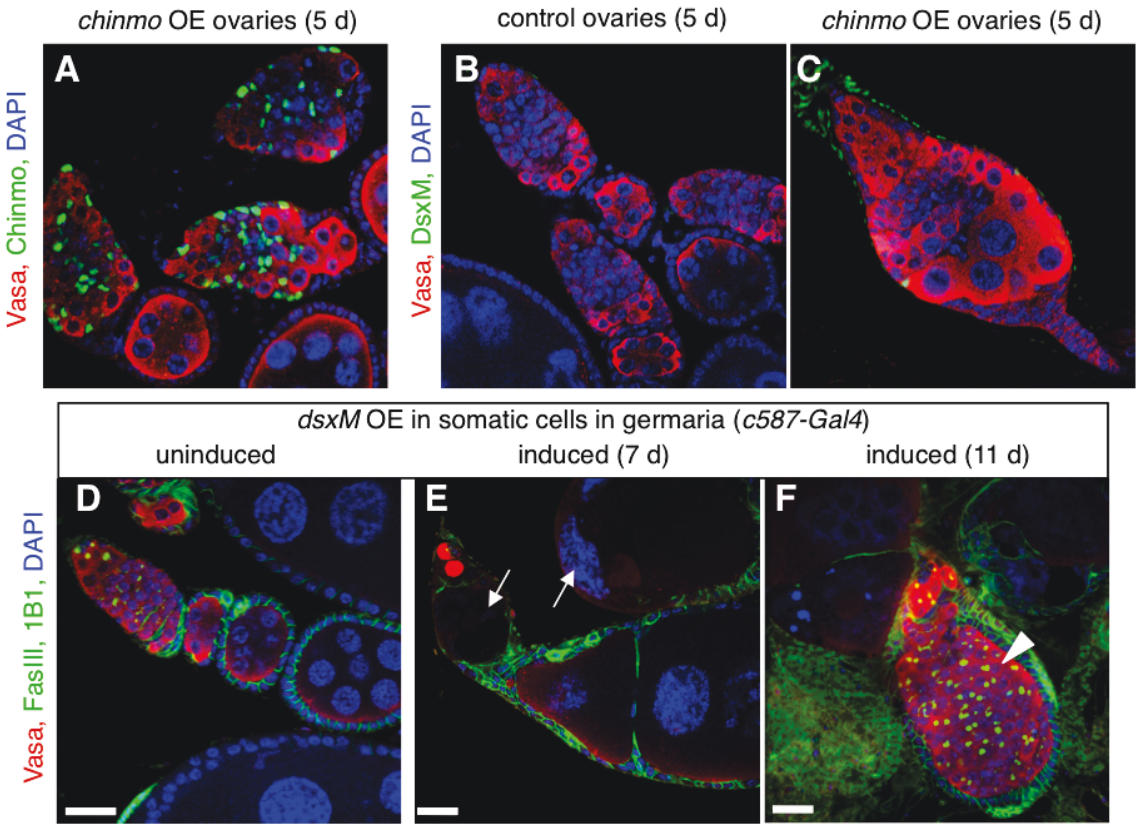

chinmo OE ovaries (5 d)
control ovaries (5 d)
chinmo OE ovaries (5 d)
A
B
C
Vasa, Chinmo, DAPI
Vasa, DsxM, DAPI
dsxM OE in somatic cells in germaria (c587-Gal4)
uninduced
induced (7 d)
induced (11 d)
D
E
F
Vasa, FasIII, 1B1, DAPI

Figure 3.4

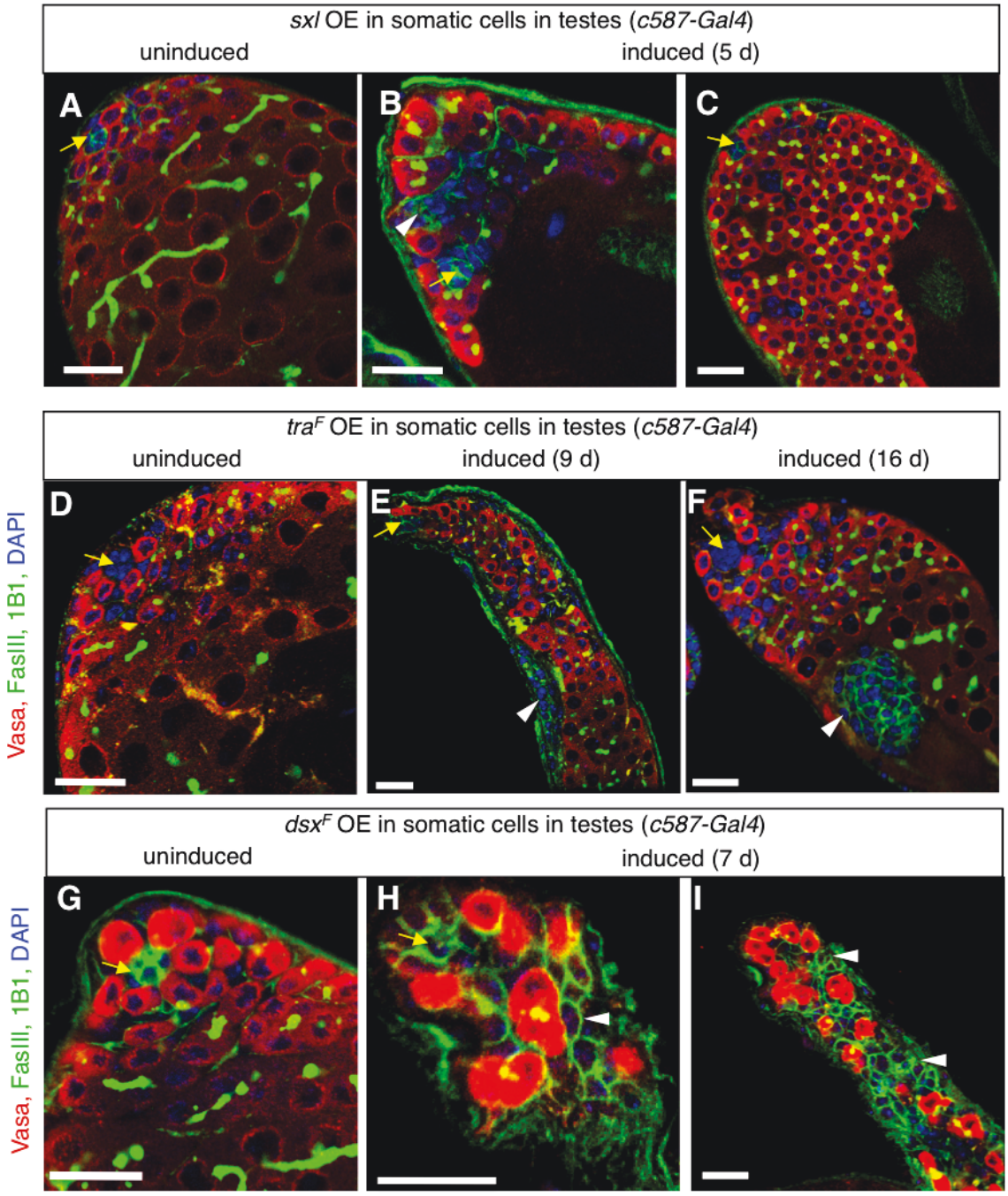

sxl OE in somatic cells in testes (c587-Gal4)
uninduced
induced (5 d)
A
B
C
traF OE in somatic cells in testes (c587-Gal4)
uninduced
induced (9 d)
induced (16 d)
D
E
F
Vasa, FasIII, 1B1, DAPI
dsxF OE in somatic cells in testes (c587-Gal4)
uninduced
induced (7 d)
G
H
I
Vasa, FasIII, 1B1, DAPI

Figure 3.5

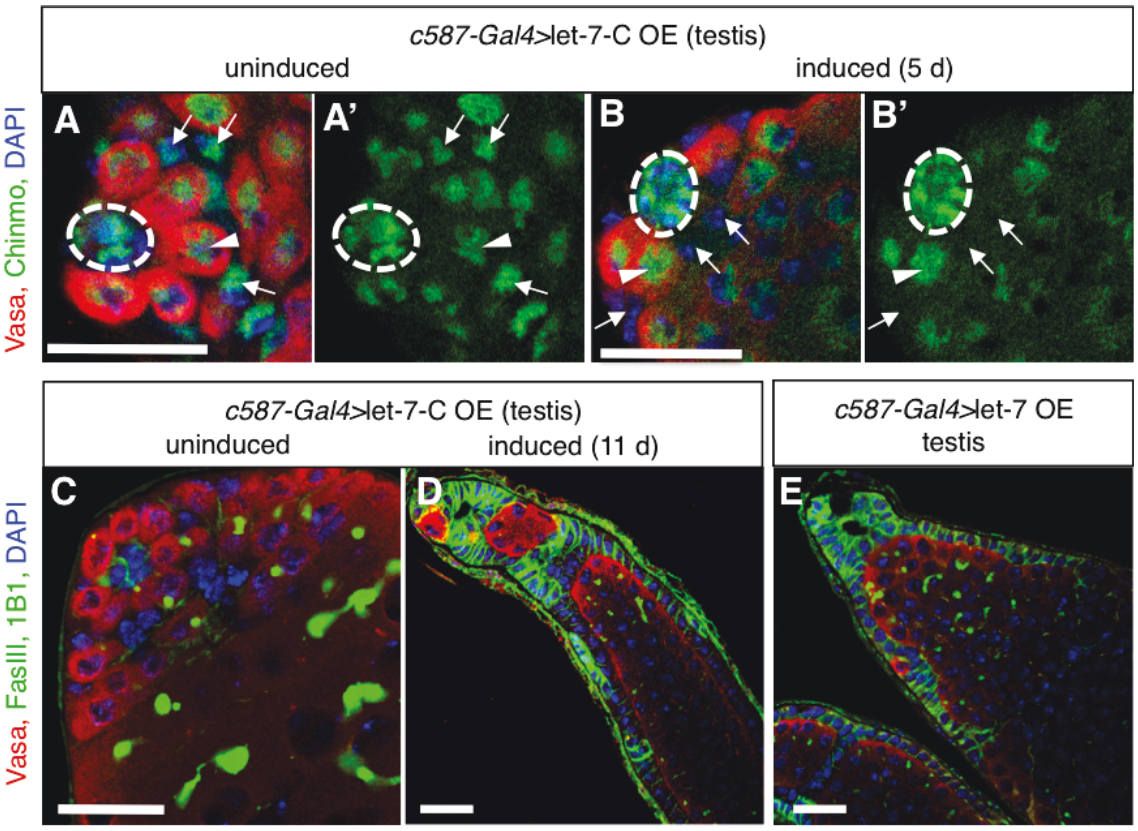

c587-Gal4>let-7-C OE (testis)
uninduced
induced (5 d)
A
A'
B
B'
Vasa, Chinmo, DAPI
c587-Gal4>let-7-C OE (testis)
uninduced
induced (11 d)
c587-Gal4>let-7 OE
testis
C
D
E
Vasa, FasIII, 1B1, DAPI

Figure 3.6

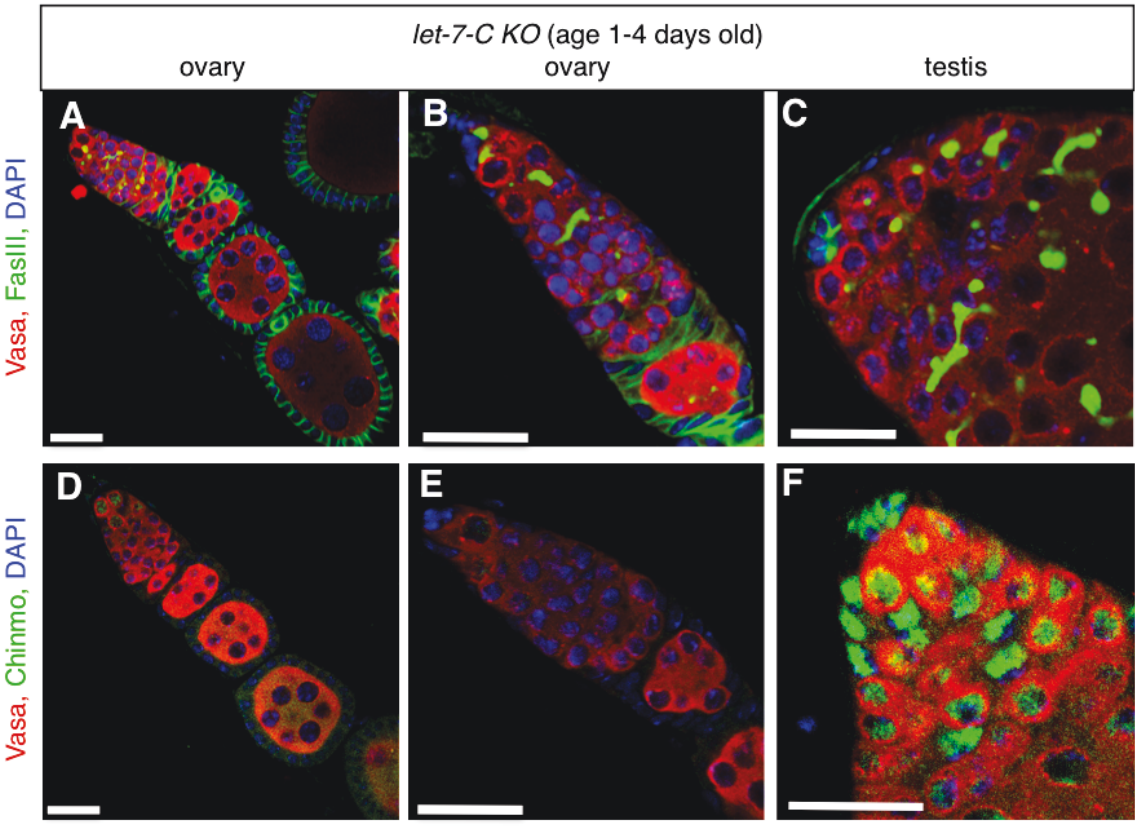

let-7-C KO (age 1-4 days old)
ovary
ovary
testis
A
B
C
D
E
F
Vasa, FasIII, DAPI
Vasa, Chinmo, DAPI

Figure S3.1

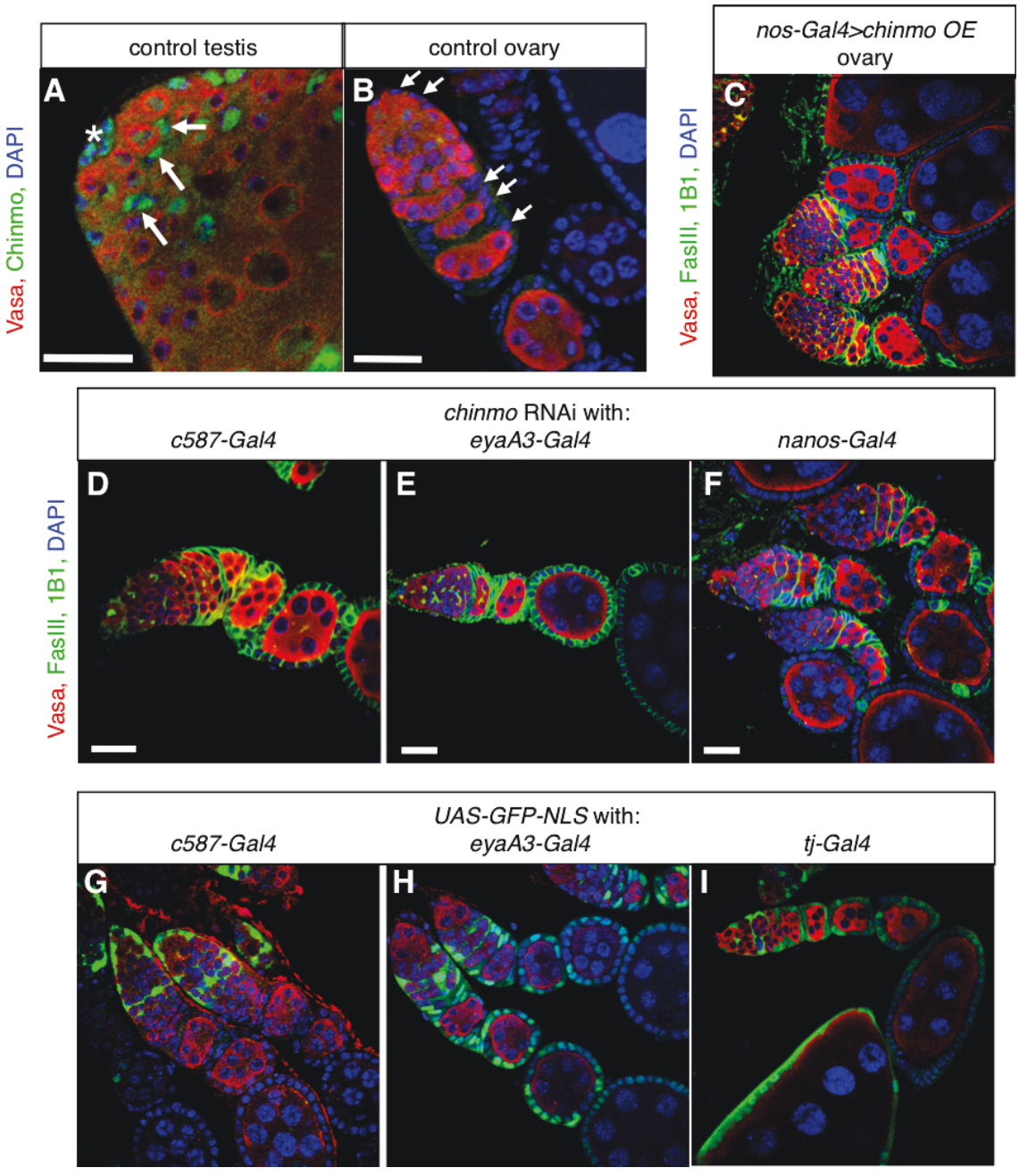

control testis
control ovary
A
B
*
Vasa, Chinmo, DAPI
nos-Gal4>chinmo OE
ovary
C
Vasa, FasIII, 1B1, DAPI
chinmo RNAi with:
c587-Gal4
eyaA3-Gal4
nanos-Gal4
D
E
F
Vasa, FasIII, 1B1, DAPI
UAS-GFP-NLS with:
c587-Gal4
eyaA3-Gal4
tj-Gal4
G
H
I

Figure S3.2

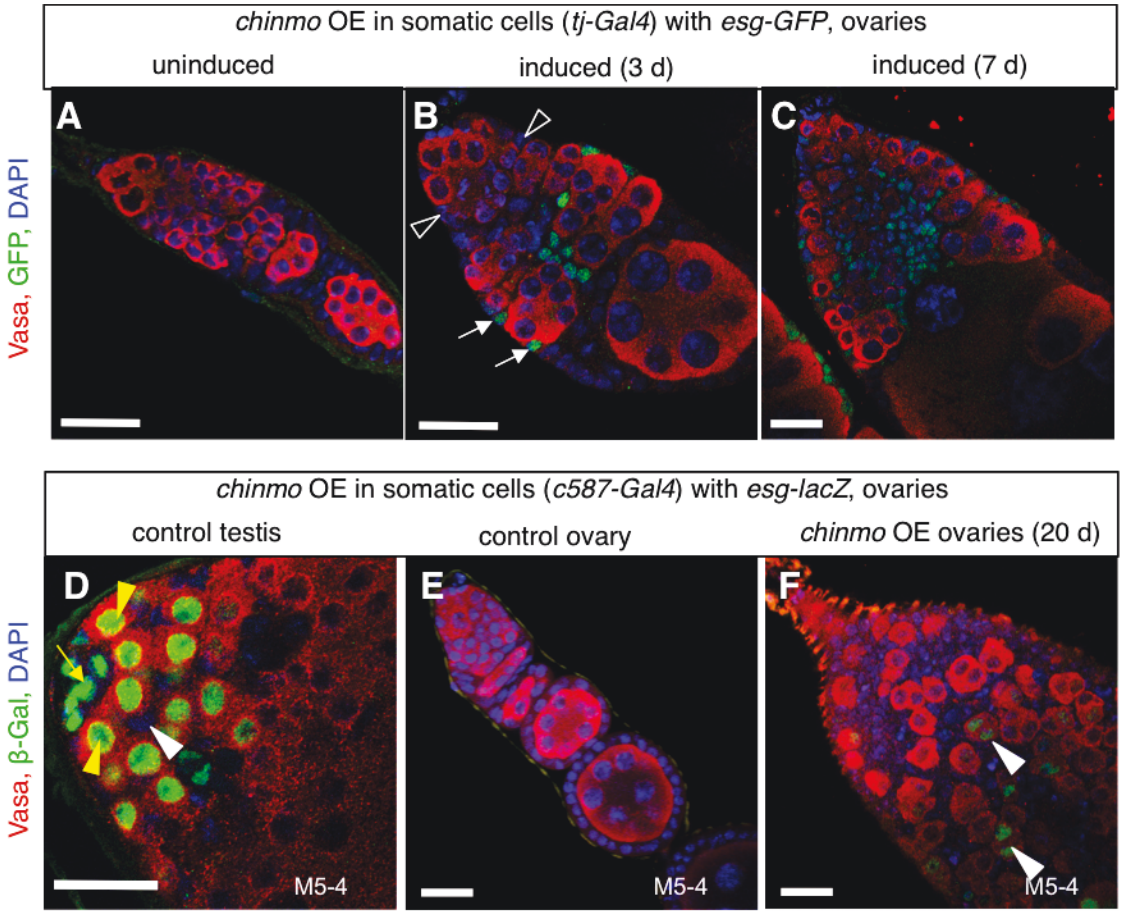

chinmo OE in somatic cells (tj-Gal4) with esg-GFP, ovaries
uninduced
induced (3 d)
induced (7 d)
A
B
C
Vasa, GFP, DAPI
chinmo OE in somatic cells (c587-Gal4) with esg-lacZ, ovaries
control testis
control ovary
chinmo OE ovaries (20 d)
D
E
F
M5-4
M5-4
M5-4
Vasa, β-Gal, DAPI

Figure S3.3

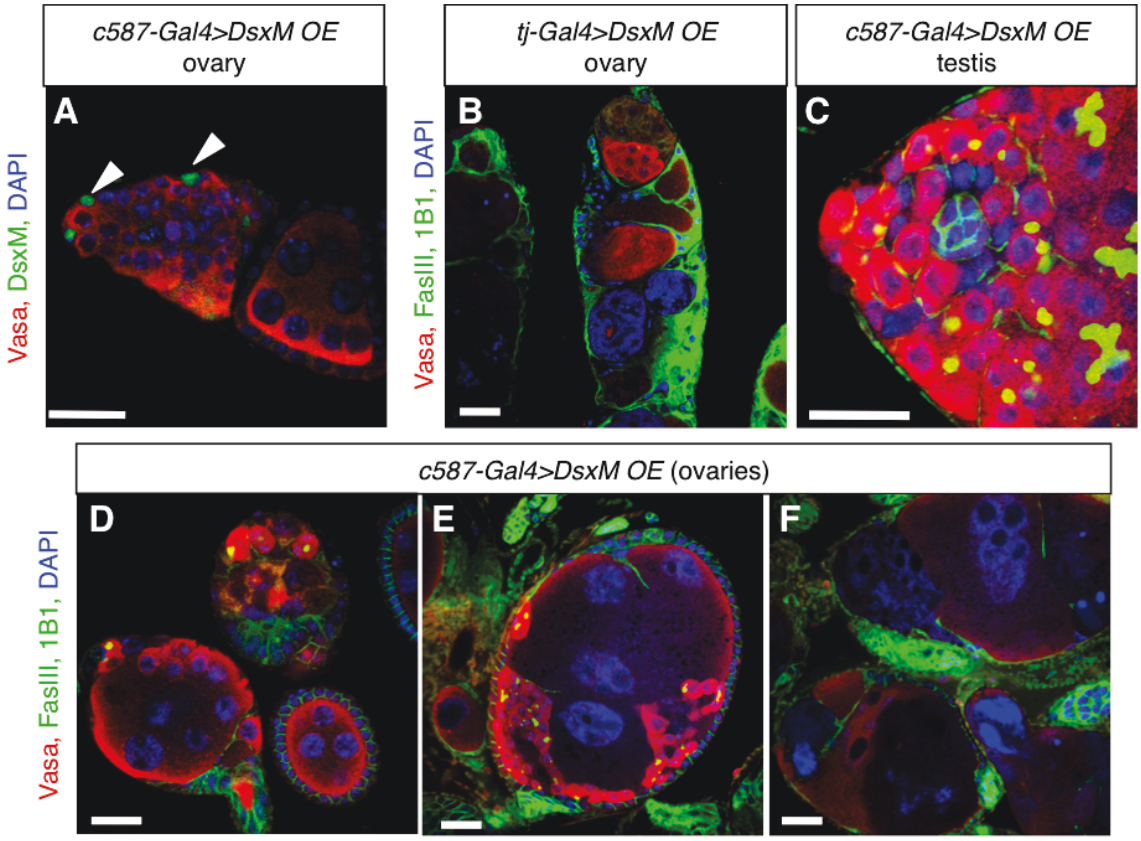

c587-Gal4>DsxM OE
ovary
tj-Gal4>DsxM OE
ovary
c587-Gal4>DsxM OE
testis
A
B
C
Vasa, DsxM, DAPI
Vasa, FasIII, 1B1, DAPI
c587-Gal4>DsxM OE (ovaries)
D
E
F
Vasa, FasIII, 1B1, DAPI

Figure S3.4

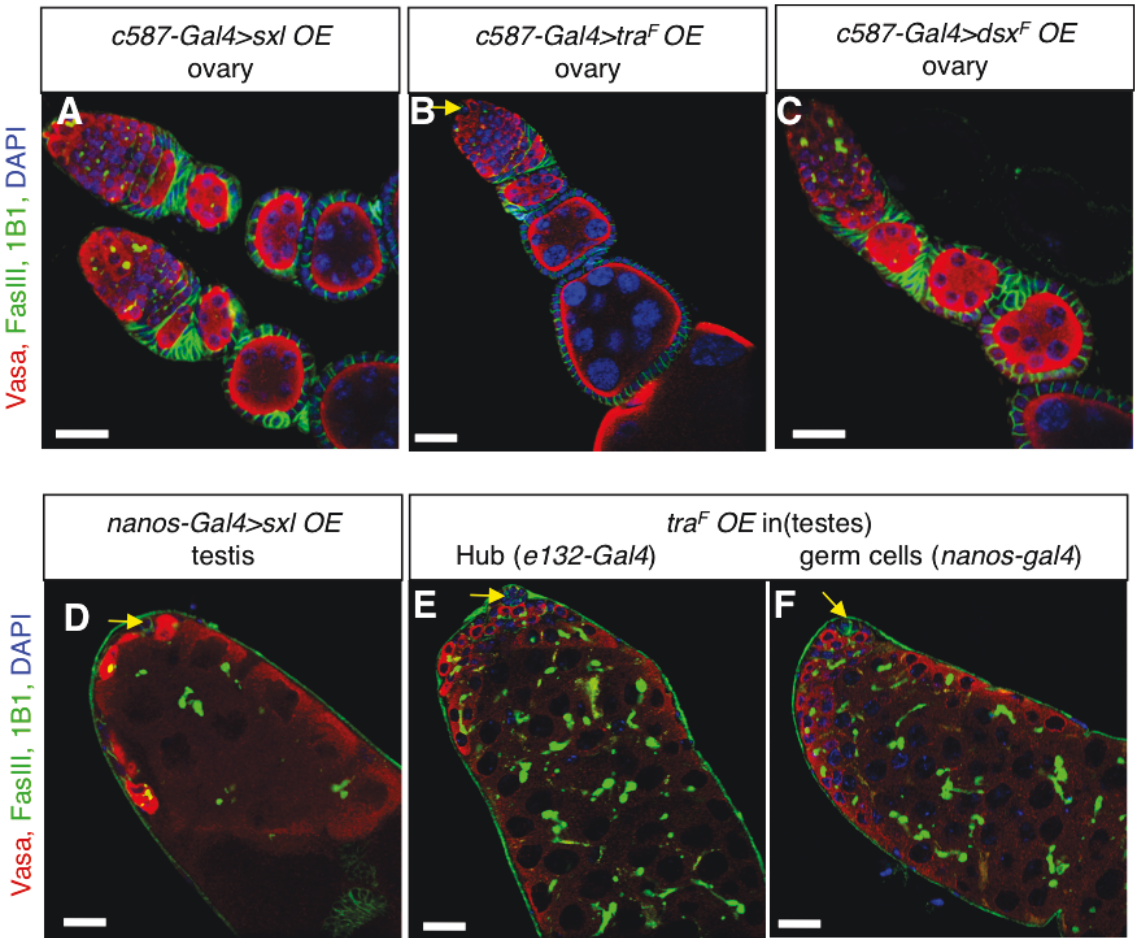
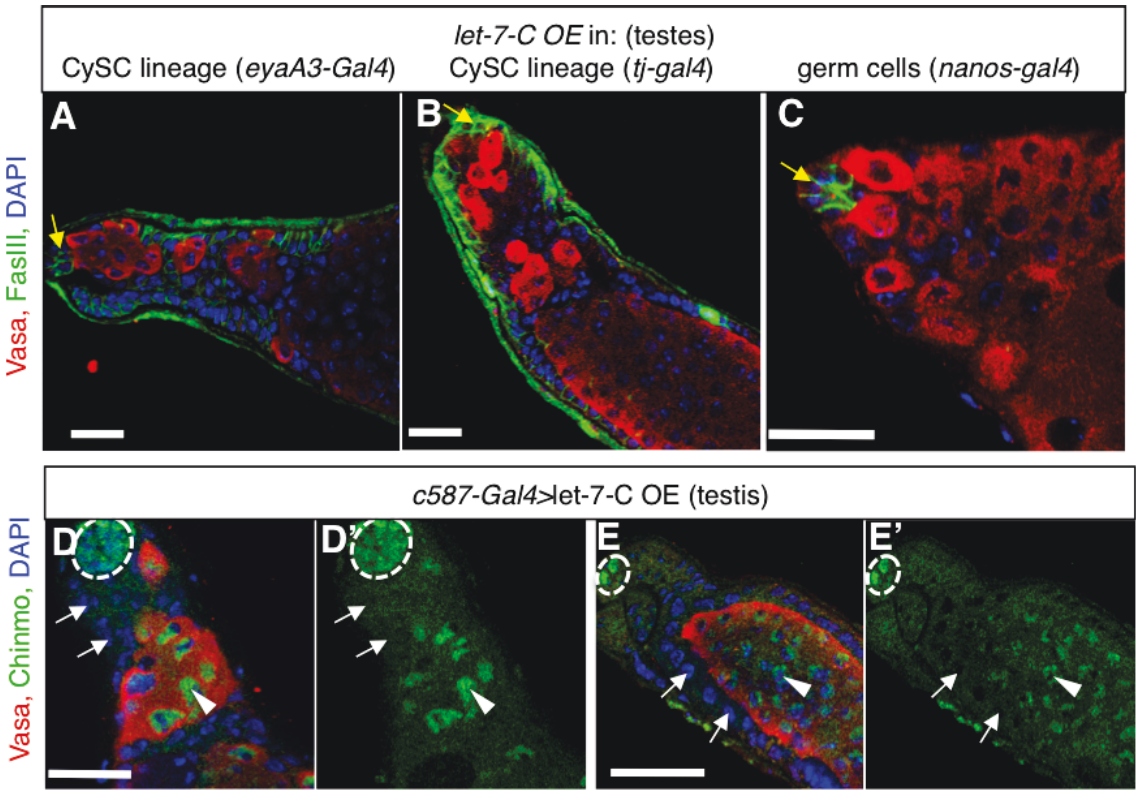

c587-Gal4>sxl OE
ovary
c587-Gal4>traF OE
ovary
c587-Gal4>dsxF OE
ovary
A
B
C
Vasa, FasIII, 1B1, DAPI
nanos-Gal4>sxl OE
testis
traF OE in(testes)
Hub (e132-Gal4)
germ cells (nanos-gal4)
D
E
F
Vasa, FasIII, 1B1, DAPI

Figure S3.5


let-7-C OE in: (testes)
CySC lineage (eyaA3-Gal4)
CySC lineage (tj-gal4)
germ cells (nanos-gal4)
A
B
C
Vasa, FasIII, DAPI
c587-Gal4>let-7-C OE (testis)
D
D'
E
E'
Vasa, Chinmo, DAPI

Chapter 4

Steroid Signaling Promotes Stem Cell Maintenance in the Drosophila Testis

This chapter is a modified version of the manuscript, "Li Y, Ma Q**,** Cherry CM, Matunis E. (2014) Steroid signaling promotes stem cell maintenance in the Drosophila Testis. *Developmental Biology* 394: 129-141."

Summary

Stem cell regulation by local signals is intensely studied, but less is known about the effects of hormonal signals on stem cells. In *Drosophila,* the primary steroid twenty-hydroxyecdysone (20E) regulates ovarian germline stem cells (GSCs) but was considered dispensable for testis GSC maintenance. Male GSCs reside in a microenvironment (niche) generated by somatic hub cells and adjacent cyst stem cells (CySCs). Here, we show that depletion of 20E from adult males by overexpressing a dominant negative form of the *Ecdysone receptor* (*EcR*) or its heterodimeric partner *ultraspiracle* (*usp*) causes GSC and CySC loss that is rescued by 20E feeding, uncovering a requirement for 20E in stem cell maintenance. EcR and USP are expressed, activated and autonomously required in the CySC lineage to promote CySC maintenance, as are downstream genes *ftz-f1* and *E75*. In contrast, GSCs non-autonomously require ecdysone signaling. Global inactivation of *EcR* increases cell death in the testis that is rescued by expression of *EcR-B2* in the CySC lineage, indicating that ecdysone signaling supports stem cell viability primarily through a specific receptor isoform. Finally, *EcR* genetically interacts with the NURF chromatin-remodeling complex, which we previously showed maintains CySCs. Thus, although 20E levels are lower in males than females, ecdysone signaling acts through distinct cell types and effectors to ensure both ovarian and testis stem cell maintenance.

Introduction

Adult stem cells, which are essential for the maintenance of many tissues, reside in niches, or local microenvironments, where distinct signals prevent their differentiation (or promote their maintenance) (Li and Xie 2005, de Cuevas and Matunis 2011). Stem cells can respond to both local and systemic signals including nutrition and hormones, which convey information about the organism's environment to the tissues and coordinate responses to physiological change (Drummond-Barbosa and Spradling 2001, Ito, Hirao et al. 2004, Li and Xie 2005, Drummond-Barbosa 2008, Hsu, LaFever et al. 2008, McLeod, Wang et al. 2010, Gancz and Gilboa 2013). Some of the best-characterized niches are found in the *Drosophila* gonads, where germline stem cells (GSCs) and supporting somatic stem cells remain active throughout adulthood, ensuring a lifetime supply of sperm or eggs (Spradling, Fuller et al. 2011). However, the role of hormonal signaling in stem cell maintenance is not fully understood, especially in the *Drosophila* testis (Gancz and Gilboa 2013).

In *Drosophila*, the steroid hormone twenty-hydroxyecdysone (20E), generated from the prohormone ecdysone, is essential for coordinating development at all stages, including embryogenesis, larval molting, puparium formation, and metamorphosis (Baehrecke 1996, Yamanaka, Rewitz et al. 2013). 20E acts by binding to a heterodimeric nuclear hormone receptor complex composed of *Ecdysone receptor* (*EcR*) and *ultraspiracle* (*usp*), which are mammalian orthologues of *farnesoid X receptor*/*liver X receptor* and *retinoid X receptor*, respectively (Hayward, Bastiani et al. 1999, King-Jones and Thummel 2005). This complex binds to specific promoter sequences, called Ecdysone Response Elements (EcREs), and can activate or repress the expression of hundreds of target genes which vary in response to the presence or absence of cell-type-specific co-activators (Tsai, Kao et al. 1999, Perera, Zheng et

al. 2005, Jang, Chang et al. 2009, Francis, Zorzano et al. 2010, Carbonell, Mazo et al. 2013) (Fig. 4.1A). Additional temporal and spatial control of 20E signaling is generated through alternative splicing of transcripts encoded by the *EcR* gene to yield three isoforms, *EcR-A*, *EcR-B1*, and *EcR-B2*; these receptors share common ligand binding domains (LBDs) and DNA binding domains (DBDs) but vary at their amino-termini. Each *EcR* isoform has a distinct expression pattern and response to 20E throughout development (Talbot, Swyryd et al. 1993).

Although ecdysone signaling has been studied primarily during metamorphosis, 20E is also present, albeit at lower levels, in adult *Drosophila* (Hodgetts, Sage et al. 1977, Handler 1982, Bownes, Dubendorfer et al. 1984, Kozlova and Thummel 2000). Adult 20E titers respond to changes in diet and environment (Riehle and Brown 1999, Tu, Yin et al. 2002) and can also be modulated genetically. In this case, however, conditional manipulation of hormone levels is necessary due to the essential roles of 20E during development. 20E feeding can also serve as a tool to increase hormone titers (Garen, Kauvar et al. 1977).

Although 20E has been shown to regulate a few aspects of adult *Drosophila* behavior including sleep and longevity, the effects of this hormone are best understood during female reproduction, where ecdysone signaling regulates multiple stages of oogenesis (Carney and Bender 2000, Ishimoto, Sakai et al. 2009, Tricoire, Battisti et al. 2009, Ishimoto and Kitamoto 2010). Oogenesis is initiated through asymmetric GSC divisions, and *EcR*, *usp*, and the ecdysone target gene and ETS-domain DNA-binding protein *Ecdysone-induced protein 74EF* (*E74)* are required directly in ovarian GSCs for their maintenance and proliferation. Both *EcR* and *E74* interact genetically with components of the Nucleosome remodeling factor (NURF) complex, suggesting that ecdysone signaling regulates GSCs by modulating their epigenetic

state (Ables and Drummond-Barbosa 2010). Ovarian GSCs are also regulated indirectly by ecdysone signaling: *EcR*, *usp*, and the ecdysone target and nuclear hormone receptor *Ecdysone-induced protein 75B* (*E75)* are required in the somatic escort cells of the ovary for GSC maintenance (Morris and Spradling 2012). Ecdysone signaling is also required for many subsequent steps in oogenesis including germline differentiation, entry into meiosis, and formation and progression of egg chambers past mid-oogenesis (Buszczak, Freeman et al. 1999, Konig, Yatsenko et al. 2011, Morris and Spradling 2012).

In contrast to the wealth of information regarding the roles of ecdysone signaling in the ovary, little is known of its requirements in male reproduction. Adult *Drosophila* males contain lower titers of 20E than females, and although the hormone has been detected in the testis (Hodgetts, Sage et al. 1977, Handler 1982, Bownes, Dubendorfer et al. 1984, Parisi, Gupta et al. 2010), ecdysone signaling was recently described as being dispensable for GSC maintenance and early germ cell development in males (Morris and Spradling 2012). However, we previously found that the NURF complex is required for stem cell maintenance in the testis (Cherry and Matunis 2010). In light of the physical and genetic interactions between NURF and ecdysone pathway components during development and oogenesis (Badenhorst, Xiao et al. 2005, Ables and Drummond-Barbosa 2010), we were prompted to look more closely at the role of ecdysone signaling in the testis stem cell niche.

The *Drosophila* testis stem cell niche resides in the testis apex, where a cluster of non-mitotic somatic cells called the hub produces signals that maintain surrounding GSCs and cyst stem cells (CySCs) (Fig. 4.1B). GSCs generate gonialblast daughters, which mitotically amplify and ultimately differentiate into sperm; CySCs produce non-mitotic daughters called cyst cells, two of which envelop each gonialblast and its descendants, supporting their

differentiation into sperm. Here, we report that ecdysone signaling pathway components are expressed and activated in CySC lineage cells and are required directly in these cells to maintain both GSCs and CySCs, which do not survive in the absence of ecdysone signaling. Moreover, we show that *EcR* interacts genetically with *Enhancer of bithorax* (*Nurf301*), a component of the NURF complex, to maintain stem cells in the testis niche. Thus, steroid signaling is required for stem cell maintenance in both the ovary and testis of *Drosophila*, where it might act in part by regulating the epigenetic state of the stem cells.

Results

Ecdysone signaling components are expressed and activated in the *Drosophila* testis

To determine whether ecdysone signaling plays a role in the adult *Drosophila* testis, we began by asking whether ecdysone receptors and downstream targets of the pathway are expressed in this tissue. We used immunostaining to determine the expression patterns of *EcR*, *usp*, and the downstream targets *broad* (*br), E75* and *ftz transcription factor 1* (*ftz-f1*) in the testis apex. We found that USP is expressed in the hub and CySC lineage cells (Fig. 4.1C), while EcR and Br are enriched in the CySC lineage (Fig. 4.1D and E). Although transcripts encoding E75 and Ftz-f1 were detected in the testis by RNA-seq (Gan, Chepelev et al. 2010), these proteins are below the level of detection via immunostaining in adult testes (although they were detected in other tissues; data not shown). Thus, several key ecdysone pathway components are present within the testis apex, and their expression is largely confined to somatic cells.

Since ecdysone pathway members are expressed in the testis apex, we next asked which cells in this tissue actively transduce ecdysone signaling. Transgenic flies containing chimeric receptors are well-established tools for detecting ecdysone receptor complex (EcR and USP) activation within tissues. These receptors contain the ligand-binding domain from either EcR or USP fused to the yeast GAL4 DNA-binding domain (*GAL4-EcR* or *GAL4-usp*) under control of a heat-inducible promoter, which allows for precise temporal control of their expression (Kozlova and Thummel 2002). Binding of GAL4-EcR or GAL4-USP to a second transgene encoding a reporter (lacZ or GFP) under control of an upstream activating sequence (UAS), which is recognized by the Gal4 DNA-binding domain, reveals cells with active ecdysone signaling. When flies carrying both transgenes are exposed to high temperature,

chimeric receptors are expressed throughout the fly; however, UAS-reporter genes are expressed only in cells containing 20E and the cognate receptor (USP or other binding partners for *GAL4-EcR*; EcR or other binding partners for *GAL4-usp*) (Fig. 4.1F) (Kozlova and Thummel 2002, Palanker, Necakov et al. 2006). We first examined testes from late 3rd instar larvae expressing GAL4-EcR, because at this stage, the stem cell niche is fully functional but the endogenous 20E levels are higher than in adults (Hardy, Tokuyasu et al. 1979, Kozlova and Thummel 2000). We observed weak GFP expression in a few hub cells and stronger expression in late cyst cells (Fig. 4.1G). However, when flies develop to adulthood and 20E titers have diminished (Schwedes and Carney 2012), GFP expression is no longer detectable within the testis (Fig. 4.1H). Therefore, we hypothesized that in larval testes, endogenous 20E levels are sufficient to induce *GAL4-EcR* activation in the somatic lineage, but in adult testes, 20E availability might be a limiting factor. To test this hypothesis, we fed exogenous 20E to adult flies containing *GAL4-EcR* and *UAS-lacZ* and then examined the reporter gene expression within the testis. We found that 20E feeding caused *GAL4-EcR* activation in the hub and CySC lineage in a pattern similar to that seen in 3rd instar larval testes in response to endogenous hormone (Fig. 4.1I). We conclude that adult hub and CySC lineage cells are competent to respond to 20E via EcR, but that the levels of 20E needed to produce a detectable signal using this reporter are insufficient when flies are fed standard food. When we repeated the 20E feeding with flies expressing *GAL4-usp*, we again saw GFP expression in the hub and late cyst cells (Fig. 4.1J). We expected to see activation of these reporters in the CySC lineage, but were surprised to find GFP expression in the hub; *GAL4-usp* requires a binding partner to function, and we did not detect endogenous EcR expression in the hub (Fig. 4.1D). Perhaps low levels of EcR are present in the hub (but undetectable by

immunostaining) and are sufficient to activate reporter gene expression. However, *usp*, unlike *EcR*, can signal through additional binding partners such as *Hormone receptor-like in 38* (*DHR38*) (Jones, Wozniak et al. 2001, Baker, Shewchuk et al. 2003); these partners, which have not been characterized in the testis, may permit activation of *GAL4-usp*. We observed that the activation of both ecdysone activity reporters was limited to only a few cells, and we suspect that this is due to a limited supply of binding partners. In support of this idea, *GAL4-usp* activation becomes detectable in almost all hub and CySC lineage cells upon co-expression of EcR (data not shown). This finding suggests that the low levels of endogenous EcR detected by immunostaining in the CySC lineage are insufficient to activate *GAL4-usp* in all cells. Similarly, expression of a constitutively active form of the EcR co-activator *taiman* (*tai*) yielded *GAL4-EcR* reporter activation in almost all hub and CySC lineage cells in the testis apex (data not shown). Taken together, our results indicate that EcR and USP can be activated specifically within hub cells and CySC lineage cells in the presence of their binding partners in both larval and adult testes, and that receptor complex activation in the adult testis is ligand-dependent.

20E is required for male germline and somatic stem cell maintenance

Since ecdysone signaling components are expressed and can be activated in the testis, we hypothesized that 20E plays a role in this tissue even though its endogenous titer is very low. To test this hypothesis, we asked whether 20E is required to maintain adult male GSCs or CySCs. To reduce the effective concentration of 20E, we used the *GAL4-EcR* and *GAL4-usp* constructs described above, which have been widely used as dominant negative (DN) receptors when overexpressed for an extended period of time (Kozlova and Thummel 2002,

Kozlova and Thummel 2003, Hackney, Pucci et al. 2007, Konig, Yatsenko et al. 2011). For example, both heat-shocked *Gal4-EcR* flies and flies expressing *UAS-EcR.B1-ΔC655.F645A*, a DN form of *EcR*, in border cells develop a similar thin eggshell phenotype (Hackney, Pucci et al. 2007). Testes from control flies, which carry the *GAL4-EcR* or *GAL4-usp* construct but are un-induced, appear normal (Figs. 4.2B and S4.1A). After extended overexpression of either construct, however, testes lose most of their GSCs, early germline cells, and CySCs (Figs. 4.2C, E and S4.1B), suggesting that signaling via 20E contributes to the maintenance of both stem cell populations in the testis. Because the endogenous titer of 20E in the adult testis is very low, we speculated that these constructs could act as DN receptors by binding with endogenous receptors and then competing with endogenous heterodimers for the limited amount of 20E, similar to a 20E "sponge" (Fig. 4.2A). To ask whether the loss of stem cells is due to reduced titers of 20E by *GAL4-EcR* or *GAL4-usp*, we repeated the above experiment but added 20E to the fly food to increase hormone levels. We expected that if 20E is no longer the limiting factor, endogenous *EcR* and *usp* should function normally; therefore, feeding 20E should rescue the phenotype caused by overexpression of *GAL4-EcR* or *GAL4-usp*. Consistent with our hypothesis, 20E feeding significantly rescued the GSC and CySC loss caused by extended overexpression of *GAL4-EcR* or *GAL4-usp* (Figs. 4.2D ,E and S4.1C). We conclude that although 20E is present only at very low levels in the testis, it is required to maintain GSCs and CySCs.

ecd plays an ecdysone-independent role in GSC and CySC maintenance

As an alternate approach to reducing ecdysteroid levels in the testis, we used a temperature-sensitive allele of *ecdysoneless* (*ecd*^{1}). This steroid-deficient fly strain has long

been used to study the effects of ecdysone signaling in *Drosophila*, but it has both ecdysone-dependent and independent functions (Garen, Kauvar et al. 1977, Gaziova, Bonnette et al. 2004, Ables and Drummond-Barbosa 2010, Claudius, Romani et al. 2014). Therefore, rescue of *ecd* phenotypes by 20E feeding is important to distinguish between these possibilities. After shifting adult ecd^{1} flies to the non-permissive temperature for 7 days, we found that their testes contained significantly fewer GSCs than un-shifted control testes. We expected that we could rescue this GSC loss phenotype by feeding 20E to the flies. However, we found that the phenotype was not rescued by 20E feeding (Fig. S4.2A-D), although the same feeding paradigm was sufficient to activate *GAL4-EcR* (Fig. 4.1I and J). We conclude that *ecd*-dependent GSC loss is caused by an ecdysone-independent role of *ecd*. Moreover, mosaic analysis revealed that *ecd* is required cell-autonomously in the GSCs and CySCs for their maintenance (Fig. S4.2E, Table S4.1). The inability of adjacent wild-type cells to compensate for loss of *ecd* function further indicates that ecdysteroid production is not the main role for *ecd* in the testis niche. We conclude that *ecd* is required to maintain GSCs and CySCs in the testis niche; however, since its requirement is independent of 20E, *ecd* is not a useful tool for studying the role of ecdysone signaling in this tissue.

EcR and *usp* are required in the CySC lineage to maintain GSCs and CySCs

Knowing that 20E is required to maintain stem cells in the testis, we next asked whether the ecdysone receptors *EcR* and *usp* are also required. Flies carrying a temperature sensitive allele of *EcR*, EcR^{A483T}, in trans with a null allele, EcR^{M554fs}, have normal numbers of GSCs and CySCs when raised at permissive temperature (Fig. 4.3B). However, after 7 days at restrictive temperature, $EcR^{A483T/M554fs}$ (EcR^{ts}) flies have significantly fewer GSCs and CySCs

than heterozygous control flies under the same conditions (Fig. 4.3A-D). In addition, we found differentiating spermatogonial cells next to the hub in 23% of mutant testes at restrictive temperature (n = 31); this phenotype, which does not occur in wild-type testes (Fig. 4.3C), is indicative of GSC depletion. The stem cell loss phenotype of EcR^{ts} testes shows that *EcR* promotes stem cell maintenance in the testis, but does not reveal which cells autonomously require *EcR,* since this mutant combination yields a global reduction in receptor activity. Since EcR and USP are undetectable in germ cells but are present in the CySC lineage, we hypothesized that these receptors are required autonomously within somatic stem cells for their maintenance. The genomic location of *usp* (on the X chromosome) and *EcR* (very close to the centromere) precludes mosaic analysis of these genes in the testis. However, RNAi-mediated knockdown is a feasible alternative. We used the CySC and early cyst-cell driver *c587-Gal4* in combination with a temperature-sensitive allele of the *Gal4* repressor *Gal80* to conditionally express transgenic RNAi or DN constructs of *EcR* or *usp* specifically in the adult testis. After 14 days of transgene induction at 29˚C, we observed a significant decrease in the number of CySCs in all four experimental genotypes (Figs. 4.4A-E, S4.3 and S4.4). Although we could not detect EcR in the hub, we did detect USP there, so we also asked whether there is a requirement for each receptor in hub cells. However, hub cells in testes containing RNAi-mediated knock down of *EcR* or *usp* in the hub were indistinguishable from those in control testes; in addition there was no significant effect on CySC numbers (Fig. S4.4). These results indicate that *EcR* and *usp* are cell-autonomously required in the CySC lineage, but not in hub cells, for CySC maintenance. After *EcR* or *usp* knockdown in the CySC lineage, we also found that the number of GSCs decreased significantly (Fig. 4.4E), which suggests that *EcR* and *usp* are required indirectly in the CySC lineage for GSC maintenance. GSCs could be lost

simply as a consequence of CySC loss, but it is also possible that they rely on ecdysone-dependent maintenance signals from CySCs. We have never observed expression or activation of ecdysone signaling pathway components in GSCs, or significant GSC loss, when *EcR* or *usp* are knocked down by RNAi in the germline (data not shown). We conclude that *EcR* and *usp* are required autonomously in the CySC lineage, and non-autonomously for GSC maintenance.

We next asked whether expression of EcR only in the CySC lineage is sufficient to rescue the stem cell loss phenotype of EcR^{ts} testes and whether the requirement of *EcR* is isoform-specific. To answer this question, we expressed each isoform (*EcR-A*, *EcR-B1*, or *EcR-B2*) independently in the CySC lineage in the EcR^{ts} mutant background. Interestingly, we found that expression of *EcR-B2*, but not *EcR-A* or *EcR-B1*, in the CySC lineage is able to fully rescue the EcR^{ts} stem cell loss phenotype (Fig. 4.4F-J). In contrast, expression of *EcR-A*, *EcR-B1*, or *EcR-B2* in hub cells did not rescue the EcR^{ts} phenotype (Fig. S4.5). These results indicate that within the CySC lineage, *EcR* is necessary for stem cell maintenance in the testis, and its requirement is specific to the *EcR-B2* isoform, which can act as a strong ligand-dependent transcriptional activator (King-Jones and Thummel, 2005).

EcR is required for cell survival in the testis

Ecdysone signaling is known to regulate apoptosis during development, and in the ovary, developing germline cysts lacking ecdysone signaling die more often than control cysts (Ables and Drummond-Barbosa 2010, Zirin, Cheng et al. 2013). Therefore, we asked whether stem cell loss in EcR^{ts} testes at restrictive temperature could be caused by increased cell death. We used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect

fragmented DNA in dying cells, and we counted the number of dying cells in testes from EcR^{ts} flies that remained at permissive temperature (control testes) or were shifted to restrictive temperature for 2 days. As expected, dying cells were rarely found within the stem cell zone (within 2 cell diameters of the hub) in control testes, but in testes at restrictive temperature we observed significantly more of them (Fig. 4.5 A, B and D). These testes also had significantly more germ cell death in the differentiating cell zone than did control testes (Fig. 4.5E). To confirm that the increase in cell death is due to dysfunction of *EcR*, we expressed the *EcR-B2* isoform in the CySC lineage in EcR^{ts} testes at restrictive temperature and found that it rescues the increased cell death phenotype (Fig. 4.5C-E). Taken together, these results suggest that *EcR-B2* in the CySC lineage is necessary for promoting cell survival in the testis stem cell niche. However, it is possible that GSCs and CySCs are also lost due to early differentiation of stem cells.

The 20E targets *E75* and *ftz-f1*, but not *br,* promote stem cell maintenance in the testis

Ecdysone signaling is mediated by multiple target genes which vary by tissue type and developmental stage (Andres and Thummel 1992). To identify potential 20E target genes in the adult testis niche, we surveyed testis RNA-seq data for the expression of known ecdysone-responsive genes (RPKM>1 in wild type testes) (Gan, Chepelev et al. 2010), espeically those with known requirements in other adult stem cell-based tissues, including the ovary and intestine (Ables and Drummond-Barbosa 2010, Gan, Chepelev et al. 2010, Morris and Spradling 2012, Zeng and Hou 2012). Using these criteria, we found three candidate ecdysone targets, *E75*, *ftz-f1* and *br*, and tested the requirement for each gene in CySC maintenance using RNAi-mediated knockdown in the CySC lineage. We found that *E75* or *ftz-f1*

knockdown causes a loss of GSCs and CySCs that is similar to the phenotype resulting from knockdown of *EcR* or *usp* (Fig. 4.6C-E). In contrast, knockdown of *br* in the CySC lineage shows no effect on stem cell maintenance even though we observed significant reduction of Br protein level in the cyst stem cell lineage, confirming the efficacy of *br* knockdown (Fig. 4.6A, B and E). Mosaic analysis of *E75* and *ftz-f1* confirmed that these two factors are cell autonomously required for CySC maintenance (Fig. 4.6F-J, Table 4.1 and 4.2). We conclude that the 20E target genes *E75* and *ftz-f1,* but not *br,* are required for CySC maintenance.

EcR **genetically interacts with** ***Nurf301*** **to maintain stem cells in the testis**

In the *Drosophila* ovary, *EcR* interacts genetically with *Nurf301*, which encodes a component of the NURF chromatin remodeling complex, to promote GSC maintenance (Ables and Drummond-Barbosa, 2010). Since we had previously found that NURF is also autonomously required to promote the maintenance of male GSCs and CySCs (Cherry and Matunis 2010), we wondered if *EcR* and *Nurf301* function together in the testis. To test this hypothesis, we asked whether reduced *Nurf301* expression levels could enhance the stem cell loss phenotype of EcR knockdown. We accomplished this by knocking down *EcR* expression specifically in the CySC lineage in a *Nurf301* heterozygous background. *Nurf301* heterozygous mutant testes are indistinguishable from wild-type testes and have normal numbers of GSCs and CySCs (Cherry and Matunis 2010). In contrast, reducing *EcR* expression in *Nurf301* heterozygous CySCs causes a significant reduction in the number of GSCs and CySCs (Fig. 4.7). This result suggests that the ecdysone signaling pathway functions together with the NURF chromatin-remodeling complex to promote stem cell maintenance in both the ovary and the testis.

Discussion

Our work shows that the steroid hormone 20E plays an important role in maintaining stem cells in the *Drosophila* testis: 20E, receptors of ecdysone signaling, and downstream targets are required directly in CySCs for their maintenance. When ecdysone signaling is lost in CySCs, GSCs are also lost, but it is unclear if their maintenance requires an ecdysone-dependent or independent signal from the CySCs. We also show that the requirement for EcR in the testis is isoform-specific: expression of *EcR-B2* in the CySC lineage is sufficient to rescue loss of GSCs and CySCs and increased cell death in *EcR* mutant testes, suggesting that there might be a temporal and spatial control of ecdysone signaling in the adult testis. In addition, we provide evidence that ecdysone signaling, as in the ovary, is able to interact with an intrinsic chromatin-remodeling factor, *Nurf301*, to promote stem cell maintenance. Therefore, our studies have revealed a novel role for ecdysone signaling in *Drosophila* male reproduction.

Hormone signaling in the ovary and testis

Although ecdysone signaling is required in both ovaries and testes for stem cell maintenance, the responses in each tissue are likely to be sex-specific. In the ovary, 20E controls GSCs directly, by modulating their proliferation and self-renewal, and it acts predominantly through the downstream target gene *E74* (Ables and Drummond-Barbosa 2010). In contrast, male GSCs require ecdysone signaling only indirectly: we found that ecdysone signaling is required in the CySC lineage to maintain both CySCs and GSCs. In a previous study, RNAi-mediated knockdown of *EcR*, *usp* or *E75* in the CySC lineage did not result in a significant loss of GSCs (Morris and Spradling, 2012); however, the number of

CySCs was not determined, and the phenotype was examined after 4 or 8 days, not 14 days as in our study. We suspect that the earlier time points used in that study may not have allowed enough time for a significant number of GSCs to be lost.

Spatial and temporal regulation of ecdysone signaling

During development, 20E is produced in the prothoracic gland (PG) and further metabolized to 20E in target tissues, but the PG does not persist into adulthood (Gilbert, Rybczynski et al. 2002, Huang, Warren et al. 2008). In adult female *Drosophila*, the ovary is a source of 20E (Schwartz, Kelly et al. 1985). In contrast, the identification of steroidogenic tissues in adult male *Drosophila* remains the subject of active investigation. The level of 20E in adult males is significantly lower than in adult females, but it can be detected in the testis (Hodgetts, Sage et al. 1977, Handler 1982, Bownes, Dubendorfer et al. 1984, Schwedes and Carney 2012). Furthermore, RNA-seq data show that *shade*, which encodes the enzyme that metabolizes the prohomone ecdysone to 20E, is expressed in the adult testis, suggesting that the adult testis may produce 20E (Petryk, Warren et al. 2003, Gan, Chepelev et al. 2010). However, the sources of 20E production in adult *Drosophila* males remain to be determined experimentally.

20E, like other systemic hormones, can have tissue-specific effects or differential effects on the same cell type as development proceeds. These differences are mediated at least in part by the particular downstream target genes that are activated in each case. For example, in female 3rd instar larval ovaries, ecdysone signaling upregulates *br* expression to induce niche formation and PGC differentiation, but *br* is not required for GSC maintenance in the adult ovary (Gancz, Lengil et al. 2011); instead, *E74* plays this role (Ables and Drummond-

Barbosa 2010). Similarly, *br* is required for the establishment of intestinal stem cells (ISCs) in the larval and pupal stages but not for ISC function in adults (Zeng and Hou 2012). Here, we show that ecdysone signaling in the adult testis is mediated by different target genes than in the ovary: *E74*, but not *E75* or *br*, regulate stem cell function in the ovary, whereas *E75* and *ftz-f1* are important for stem cell maintenance in the testis. Since E75 is itself a nuclear hormone receptor that responds to the second messenger nitric oxide (Reinking, Lam et al. 2005, Caceres, Necakov et al. 2011), it will be interesting to know whether E75's partner DHR3 also plays a role in CySCs. An intriguing question for future studies will be how different ecdysone target genes interact with the various signaling pathways that maintain stem cells in the ovary or testis.

Environmental changes, stem cells and hormonal signals

Since 20E levels can actively respond to physiological changes induced by environmental cues, it is possible that the effect of 20E on testis stem cell maintenance might reflect changes in diet, stress, or other environmental cues. For example, in *Aedes aegypti*, ecdysteroid production in the ovary is stimulated by blood feeding and this is an insulin-dependent process (Riehle and Brown 1999). In *Drosophila*, ecdysone signaling is known to interact with the insulin pathway in a complex way. Ovaries from females with hypomorphic mutations in the insulin-like receptor have reduced levels of 20E (Tu, Yin et al. 2002). Furthermore, ecdysone signaling can directly inhibit insulin signaling and control larval growth in the fat body (Colombani, Bianchini et al. 2005). Thus, ecdysone signaling may interact with insulin signaling during testis stem cell maintenance. Previously, it was shown that GSCs in the ovary and testis can respond to diet through insulin signaling, which is

required to promote stem cell maintenance in both sexes (Drummond-Barbosa and Spradling 2001, Flatt, Min et al. 2008, Ueishi, Shimizu et al. 2009, McLeod, Wang et al. 2010, Wang, McLeod et al. 2011, Roth, Chiang et al. 2012). It is possible that diet can affect 20E levels and thus regulate stem cell maintenance. In addition to diet, stress can also affect 20E levels, as is the case in *Drosophila virilis*, where 20E levels increase significantly under high temperature stress (Rauschenbach, Sukhanova et al. 2000). A similar effect has been found in mammals, where the steroid hormone cortisol is released in response to psychological stressors (McGaugh 2004, Burke, Davis et al. 2005). Finally, 20E levels are also influenced by mating. In *Anopheles gambiae*, males transfer 20E to blood-fed females during copulation, which is important for egg production (Baldini, Gabrieli et al. 2013). In female *Drosophila*, whole body ecdysteroid levels also increase after mating (Harshman, Loeb et al. 1999). Studying the roles of hormonal signaling in mediating stem cell responses to stress and other environmental cues will be an exciting topic for future studies. From our work it is now clear that, as in mammals, steroid signaling plays critical roles in adult stem cell function during both male and female gametogenesis.

Experimental Procedures

Fly stocks and cultures

Fly stocks were raised at 25˚C on standard molasses/yeast medium unless otherwise indicated. The following fly stocks were used: *c587-Gal4* (Kai and Spradling 2003), *E132-Gal4* (from H. Sun), *w;; Nurf301^{2}/TM6B, Tb* and *w;; Nurf301^{3}/TM3, Ser* (from P. Badenhorst), *ftz-f1^{ex7} FRT2A* (from C. Dauphin-Villemant), *E75$^{\Delta 51}$ FRT80B (*from D. Drummond-Barbosa*), and ecd² FRT2A* (from M. Jindra). Other fly stocks came from the Bloomington Drosophila Stock Center (BDSC) or Vienna Drosophila RNAi Center (VDRC).

Immunofluorescence microscopy

Testes were dissected, fixed, and stained as described previously (Matunis 1997). The following antibodies were used: rabbit anti-Vasa (d-260) and goat anti-Vasa (dN-13) (Santa Cruz Biotechnology, 1:400); chicken anti-Vasa (from K. Howard, 1:5000); rabbit anti-GFP (Torrey Pines Biolabs, 1:10,000); chicken anti-GFP (Abcam, 1:10,000); mouse anti-β-Galactosidase (Promega, 1:1000); mouse 1B1 (1:25), mouse anti-Armadillo (N2 7A1; 1:50), mouse anti-EcR (DDA2.7; 1:50), mouse anti-EcR (Ag10.2; 1:50), and mouse anti-Broad-core (25E9.D7; 1:50) (Developmental Studies Hybridoma Bank at the University of Iowa); rabbit anti-ZFH1 (from R. Lehmann, 1:5000); guinea pig anti-ZFH1 (from J. Skeath; 1:1000); guinea pig anti-Tj (from D. Godt, 1:4000); and mouse anti-USP (from D. Montell, 1:20)(Christianson, King et al. 1992). Alexa fluor-conjugated secondary IgG (H+L) antibodies were diluted at 1:200 for 568 and 633 conjugates and 1:400 for 488 conjugates. Secondary antisera were: goat anti-rat 488, goat anti-rabbit 488 and 568, goat anti-mouse 488, 568 and 633, goat anti-chicken 488 and 568, and goat anti guinea-pig 568 and 633 (Molecular

Probes/Invitrogen). DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1 mg/ml. Fixed testes were mounted in Vectashield (Vector Labs) for imaging.

Analysis of confocal images

Confocal images were obtained with a Zeiss LSM 5 Pascal or a Zeiss LSM 510 Meta microscope and were collected as serial confocal sections at similar detection settings unless otherwise noted. Images were analyzed using the Zeiss LSM Image Browser software or Zen 2009 Light edition software. GSCs were scored as Vasa-positive cells (with a spherical fusome where specified) making contact with the hub. CySCs were scored as Zfh1-positive cells (Leatherman and Dinardo 2008), with medium to strong staining according to the rainbow indicator in the Zeiss Pascal software. All graphs were created using Prism 5 (GraphPad Software, Inc.). Statistical analysis of stem cell number was performed using Prism 5. Student's T-test was used to compare two populations, and unpaired ANOVA analysis was used to compare three or more populations.

20E feeding experiment

20E (Sigma-Aldrich) was dissolved in 10% ethanol to prepare a 25 mM stock solution. To visualize reporter activity: Adult males with the genotype *hs-EcR-LBD-GAL4; UAS-stinger* (or *UAS-lacZ*) or *hs-usp-LBD-GAL4; UAS-stinger* (or *UAS-lacZ*) were heat shocked 3 x 30 minutes at 37˚C and then placed in vials containing normal food covered with a piece of filter paper soaked with 100-150 ul of 1mM 20E (diluted in apple juice) plus green food coloring (McCormick, 1:50). A small hole was cut out of the filter paper to give the flies

access to the normal food below. After 1 day, flies with green guts (indicating that they had ingested the 20E) were dissected and stained with GFP or lacZ antibody.

To use reporters as dominant negative constructs: Adult males with the genotype *hs-EcR-LBD-GAL4; UAS-stinger*, or *hs-usp-LBD-GAL4; UAS-stinger,* or *hs-ECR-LBD-Gal4,* or *hs-usp-LBD-Gal4* were heat shocked twice everyday for 30 minutes each time (once in the early morning and once at night) for 4-5 days. For 20E rescue experiments, flies were placed in vials containing 20E (as described above) after each heat shock and dissected one day after the last heat shock. Flies were fed an equivalent concentration of ethanol dissolved in apple juice as a control.

Loss-of-Function Experiments

To assay the effect of loss of *ecd* on stem cell maintenance, 0-5 day old ecd^{1} males raised at 18˚C were shifted to non-permissive temperature (29˚C) for 7 days, and then testes were dissected and analyzed. *y w* males were processed in parallel as controls. To assay whether 20E feeding can rescue the ecd^{1} testis phenotype, we fed flies with 0.1 mM or 1 mM 20E using the method described for the 20E feeding experiment. Flies were fed an equivalent concentration of ethanol dissolved in apple juice as a control.

To assay the effect of loss of *EcR* on stem cell maintenance in adult testes, EcR^{M554fs}*/SM6b* (null allele) and EcR^{A483T}*/SM6b* (temperature sensitive allele) flies were crossed at permissive temperature (18˚C) and shifted to non-permissive temperature (31˚C) for 7 days, and testes were then dissected and analyzed. Heterozygous sibling males were processed in parallel as controls.

Temperature sensitive EcR rescue experiment

UAS-EcR.A, UAS-EcR.B1, and *UAS-EcR.B2* constructs were driven by *c587-Gal4* (cyst lineage) or *hh-Gal4* (BDSC 45546; hub cells) in the temperature sensitive *EcR* mutant background *(EcR^{M554fs}/ EcR^{A483T})*. *UAS-GFP-nls* was used as a control. Flies were grown at 18˚C and transferred to 31˚C as adults to induce expression of the UAS constructs.

RNAi and dominant negative (DN) knockdown experiments

The following RNAi or DN constructs were used for cell type-specific knockdown of ecdysone pathway components:

Gene	Genotype	Stock number
EcR	*UAS-EcR-RNAi*	VDRC 37058
	UAS-EcR-RNAi	BDSC 9726
	UAS-EcR.B1-ΔC655.F645A	BDSC 6869
	UAS-EcR.B1-ΔC655.W650A	BDSC 6872
	UAS-EcR.A.F645A	BDSC 9450
	UAS-EcR.A.W650A	BDSC 9451
	UAS-EcR.B2.F645A	BDSC 9450
USP	*UAS-USP-RNAi*	VDRC 16893
	UAS-USP-RNAi	BDSC 27258
E75	*UAS-E75-RNAi*	VDRC *44851*
ftz-f1	*UAS-ftz-f1-RNAi*	VDRC *108995*
	UAS-ftz-f1-RNAi	BDSC *27659*
br	*UAS-br-RNAi*	BDSC *27272*

Male flies carrying these constructs were crossed to females with the genotype *c587-Gal4; tubGAL80^{ts} (cyst lineage) or E132-Gal; tubGAL80^{ts} (hub cells) at* 18˚C. Males were shifted to 29˚C upon eclosion and dissected after 1-5 days. *UAS-GFP RNAi* (BDSC 9330) was used as a control for RNAi experiments and *UAS-GFP* (BDSC 4776) as a control for DN experiments. Flies carrying UAS constructs alone, without a driver, were processed in parallel to check for leakiness of each UAS construct. To look for genetic interaction between ecdysone signaling and NURF, we expressed *UAS-EcR-RNAi* (BDSC 37058) in the CySC lineage in a *Nurf301^{3}* or *Nurf301^{2}* heterozygous background. *UAS-GFP-RNAi* was used as a control for this experiment.

Mosaic analysis

Negatively marked clones were induced using the FLP, FRT-mediated mitotic recombination technique (Xu and Rubin 1993) in flies of the genotype: *y w, P[hs-FLP]/Y; P[Ubi-GFP.nls] P[w^{+} FRT]2A /ftz-f1^{ex7} P[w^{+} FRT]2A* or *y w, P[hs-FLP]/Y; P[Ubi-GFP.nls] P[w^{+} FRT]2A /ecd^{2} P[w^{+} FRT]2A* or *y w, P[hs-FLP]/Y; P[Ubi-GFP] P[neoFRT]80B/E75$^{\Delta 51}$ P[neoFRT]80B ry^{506}*. Control clones were induced in *y w, P[hs-FLP]/Y; P[Ubi-GFP.nls] P[w^{+} FRT]2A/P[w^{+} FRT]2A* or *P[hs-FLP]/Y; P[Ubi-GFP] P[neoFRT]80B/P[neoFRT]80B ry^{506}*. GSC clones were identified as cells that were Zfh1-negative, GFP-negative, and making broad contact with the hub. CySC clones were identified as cells that were Zfh1-positive, GFP-negative, and within 2 cell diameters of the hub.

Positively marked clones were induced using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo 1999) in flies of the genotype *y w, P[hs-FLP],*

P[tub-Gal4] P[UAS-CD8-GFP]; P[tub-Gal80] P[w+ FRT]2A/ftz-f1^{ex7} P[w+ FRT]2A. Control clones were induced in *y w, P[hs-FLP], P[tub-Gal4] P[UAS-CD8-GFP]; P[tub-Gal80] P[w^{+} FRT]2A/P[w^{+} FRT]2A* (Wang and Struhl 2004) (a gift from G. Struhl). CySC clones were identified as cells that were Zfh-1 positive, GFP-positive, and within 2 cell diameters of the hub.

To induce clones, 0-5 day old males were heat shocked for 3 x 30 minutes at 37˚C separated by 30-minute intervals at 25˚C. Flies were kept at 25˚C for 2, 4, 8, or 10 days after clone induction (ACI) before dissection.

Apoptosis detection

Cells undergoing apoptosis were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Chemicon International) as described (Sheng, Brawley et al. 2009). TUNEL-positive stem cells and early daughters were identified by the position of their nuclei (within two cell diameters of the hub). TUNEL-positive spermatogonia were identified as spots with a diameter greater than 5 μm and located more than two cell diameters from the hub.

Author Contributions

Y.L., Q.M., C.C. and E.M. designed the experiments; Y.L., Q.M., and C.C. performed the experiments; Y.L. and Q.M. analyzed the data; Y.L. and E.M wrote the manuscript.

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Figure Legends

Figure 4.1: Ecdysone signaling components are expressed and activated in the *Drosophila* testis niche.

(A) Diagram of the *Drosophila* testis. Around 10 GSCs (3 shown, pink) are attached to the hub. GSCs divide asymmetrically to produce daughter gonialblasts (GB) that are displaced from the hub. GBs go on to form spermatogonial cysts. Fusomes (red) are spherical in GSCs and branched in spermatogonia. Approximately 2 CySCs (blue) flank each GSC and contact the hub with cytoplasmic extensions. CySCs divide to produce cyst cell daughters; two envelop each GB and its descendants. **(B)** Diagram of the *Drosophila* ecdysone pathway. 20E (blue dots) activates this pathway by binding to a heterodimer composed of EcR and USP. Both EcR and USP contain a LBD that can bind 20E and a DBD that can recognize the EcRE and regulate downstream gene expression (pink dots). **(C-E)** Testes from adult *y w* flies stained with germline marker anti-Vasa (red), DNA stain DAPI (blue), and antibodies (green) against: **(C)** USP (hub and CySC lineage); **(D)** EcR (CySC lineage); or **(E)** ecdysone signaling target Br (CySC lineage). Insets show green channel alone. **(F)** Diagram of the *GAL4-EcR* reporter construct, which is composed of the LBD from EcR fused to the DBD from Gal4 and is under control of the hsp-70 promoter. When expressed at low levels, this reporter shows where the pathway can be activated: in the presence of 20E and EcR's binding partners, Gal4 is activated and induces expression of *UAS-lacZ* or *UAS-GFP* (green dots). A similar *GAL4-usp* construct (not shown) is activated by ecdysone and USP's binding partners. **(G)** Late 3rd instar larval testis carrying the *GAL4-EcR* reporter and stained with DAPI (blue), anti-Vasa (red), and anti-GFP (green). Without 20E feeding, endogenous 20E drives GFP expression in the larval hub and CySC lineage. Inset shows green channel alone. **(H-J)** Adult testes stained

with DAPI (blue), somatic cell marker anti-Tj (red), and anti-lacZ (green). Without 20E feeding **(H)**, adult testes carrying the *Gal4-EcR* reporter (or *Gal4-usp* reporter, not shown) do not express lacZ. After adult flies carrying the *Gal4-EcR* reporter **(I)** or *Gal4-usp* reporter **(J)** are fed 1 mM 20E overnight, testes express lacZ in the hub and CySC lineage. Hub, asterisk or arrow; CySC lineage cells, arrowhead. Scale bar in J, for all panels, = 20 μm.

Figure 4.2: 20E hormone is required for stem cell maintenance

(A) Diagram showing how *Gal4-EcR* or *Gal4-usp* can act as dominant negative constructs (20E "sponges"): when expressed at high levels, they bind with endogenous receptors, compete for endogenous 20E and reduce its effective concentration, thus preventing endogenous EcR or USP from functioning normally (Hackney et al. 2007). (B-D) Testes from adult flies carrying *Gal4-EcR* stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green; CySCs and their immediate daughters), anti-Hts/1B1 (white; fusomes), and anti-Arm (white; hub cells). Before overexpression (B), testes look normal; after heat-shock induced overexpression of *Gal4-EcR* (C), GSCs and CySCs are lost; feeding 20E to adult flies rescues the loss (D). Scale bar in D, for B-D, = 20 μm. (E) Bar graphs showing number of GSCs or Zfh1-positive cells per testis for this experiment. Data are represented as mean ± standard error of the mean (SEM). ** P-value < 0.005; *** P-value < 0.0005.

Figure 4.3: *EcR* is required in the testis to maintain GSCs and CySCs.

Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. **(A)** *EcR* heterozygous mutant testes look wild-type after 7 days at 31˚C. $EcR^{A483T/M554fs}$ mutant testes look normal at permissive temperature **(B)** but lose GSCs

and CySCs after 7 days at restrictive temperature **(C)**. At restrictive temperature, spermatogonial cysts are sometimes found touching the hub; a 8-cell cyst (identified by elongated fusome) is outlined. Hub, asterisk. Scale bar in C, for A-C, = 20 μm. **(D)** Bar graphs showing number of GSCs or Zfh1-positive cells per testis for this experiment. Data are represented as mean ± SEM. *** P-value < 0.0005.

Figure 4.4: *EcR* and *usp* are required in the CySC lineage to maintain CySCs and GSCs. **(A-D, F-I)** Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. **(A)** Mock disruption of GFP by RNAi in the CySC lineage does not affect GSC or CySC maintenance. **(B-D)** Disruption of EcR by RNAi **(B)** or DN **(C)** or disruption of USP by RNAi **(D)** in the CySC lineage causes loss of GSCs, early germline cells, and CySCs. **(E)** Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for the experiments depicted in panels A-D. **(F)** Testes from *c587-Gal4; $EcR^{A483T/M554fs}$; TM6B/+* flies lose GSCs and CySCs at restrictive temperature (similar to Fig. 4.3C). Expression of *UAS-EcR-B2* **(I)** but not *UAS-GFP* **(G)** or *UAS-EcR-A* **(H)** in the CySC lineage is able to rescue the stem cell loss phenotype in EcR^{ts} testes. Outlined cells are differentiated spermatogonia near the hub. Hub, asterisk. Scale bars in D, for A-D; in I, for F-I = 20 μm. **(J)** Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for the experiments depicted in panels F-I. In (E) and (J), data are represented as mean ± SEM. ** P-value < 0.005; *** P-value < 0.0005.

Figure 4.5: *EcR* is required for cell survival in the testis.

Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1, and TUNEL (green) to visualize apoptotic cells. *c587-Gal4; $EcR^{A483T/M554fs}$; TM6B/+* testes contain more

TUNEL-positive cells at restrictive temperature **(B)** than at permissive temperature **(A)**. TUNEL-positive cells are rarely found within 2 cell diameters of the hub under normal conditions, but their number increases at restrictive temperature (arrowhead) suggesting that *EcR* is required for early cell survival in the testis. **(C)** Expression of *UAS-EcR.B2* in the CySC lineage is able to rescue the increased cell death phenotype in the EcR^{ts} testes. Hub, asterisk. Scale bar in C, for A-C, = 20 μm. **(D, E)** Column scatter graphs showing the number of TUNEL-positive cells within 2 cell diameters of the hub (D) and the number of TUNEL-positive germ cells (diameter > 5 μm) (E) per testis for these experiments. Bars indicate mean ± SEM. * P-value < 0.05; ** P-value < 0.005.

Figure 4.6: *E75* and *ftz-f1*, but not *br*, are potential ecdysone targets that regulate stem cell maintenance in the testis.

(A, B) Testes from adult flies stained anti-Vasa (red), DAPI (blue), anti-Br (green). Disruption of Br by RNAi in the CySC lineage can effectively reduce Br level by immunostaining, but it does not cause GSC or CySC loss. **(C, D)** Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. Disruption of *E75* **(C)** or *ftz-f1* **(D)** by RNAi in the CySC lineage causes CySC and GSC loss. **(E)** Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for these experiments. Data are represented as mean ± SEM. *** P-value < 0.0005. **(F-I)** Testes from adult flies stained with anti-GFP (green), DAPI (blue), anti-Zfh1 (red). $ftz\text{-}f1^{ex7}$ CySC (GFP positive, Zfh1 positive) and cyst clones (GFP positive, Zfh1 negative) are induced at a similar rate but lost faster than wild type clones. ACI, after clone induction.

Figure 4.7: *EcR* genetically interacts with *Nurf301* to maintain stem cells in the testis.
Induction of *EcR RNAi* (7 days at 29˚C) in the CySC lineage using *c587-Gal4* driver causes GSC and CySC loss; in a $Nurf301^{3}$/+ heterozygous background, the loss is enhanced. Data are represented as mean ± SEM. * P-value < 0.05; *** P-value < 0.0005.

Figure S4.1: Ecdysone hormone is required for stem cell maintenance.
(A-C) Testes from adult flies *Gal4-usp* stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. Before overexpression **(A)**, testes look normal; after heat-shock induced overexpression of *Gal4-usp* **(B)**, GSCs and CySCs are lost; feeding 20E to adult flies rescues the loss **(C)**. Scale bar in C, for A-C, = 20 μm.

Figure. S4.2 : *Ecdysoneless* (*ecd*) has ecdysone-independent functions in the testis.
(A-C) Testes from adult temperature sensitive ecd^{1} flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. **(A)** At permissive temperature (18˚C), testes appear normal. At restrictive temperature (29˚C), testes show GSC loss **(B)** and cannot be rescued by 20E feeding **(C)**. Scale bar in C for A-C = 20 μm. **(D)** Bar graph showing number of GSCs per testis for this experiment. Data are represented as mean ± SEM. **(E)** Negatively marked clonal analysis shows that ecd^{2} (null allele) stem cell clones are lost faster than control clones, which indicates that *ecd* is required cell autonomously in the GSCs and CySCs for their maintenance.

Figure S4.3: Different alleles of *EcR RNAi*, *EcR DN* or *usp RNAi* lines causes stem cell loss.

(A) Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for *EcR* knockdown experiment using different alleles of *EcR RNAi* or *DN*. **(B)** Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for *usp* knockdown experiment using different alleles of *usp RNAi*. Data are represented as mean ± SEM. *** P-value < 0.0005.

Figure S4.4: *EcR* and *usp RNAi* construct has leaky expression.

(A) Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for *EcR RNAi* with or without driver. *EcR RNAi* without driver causes similar stem cell loss as RNAi driven by *E132-Gal4* driver, but is less severe than RNAi driven by *c587-Gal4* driver. **(B)** Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for *usp RNAi* with or without driver. *usp RNAi* without driver causes stem cell loss but is less severe than RNAi driven by *c587-Gal4* driver, but not RNAi driven by *E132-Gal4* driver. Data are represented as mean ± SEM. * P-value < 0.05, ** P-value < 0.005, *** P-value < 0.0005.

Figure S4.5: *EcR* is not required in the hub cells to maintain CySCs and GSCs.

(A-B) Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-GFP (green) **(A)** *c587-Gal4* driver drives the expression of GFP in the CySC lineage. **(B)** *hh-Gal4* driver drives the expression of GFP in the hub cells. **(C)** Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1. Expression of *UAS-EcR.B2* in the hub cells cannot rescue the stem cell loss phenotype in EcR^{ts} testes. Hub, asterisk. Scale bar in C, for A-C, = 20 μm. **(D)** Bar graphs showing the number of Zfh1-positive cells or GSCs per

testis for EcR hub rescue experiment. Data are represented as mean ± SEM. *** P-value < 0.0005 .

Table 4.1. *ftz-f1* is required cell autonomously for GSC and CySC maintenance

Part A ftz-f1 negative clonal analysis

Genotype	0d ACI	2dACI	6dACI	8dACI
Percentage of testis with CySC clones				
ftz-f1^{ex7} FRT2A	17% (3/18)	17% (7/30)	7% (2/30)	5% (1/20)
Ctrl FRT2A	0% (0/17)	77% (26/34)	40%(6/15)	35% (10/29)

Part B *ftz-f1* MARCM

Genotype	0d ACI	2d ACI	4d ACI	6d ACI	8d ACI
Percentage of testes with CySC clones					
ftz-f1^{ex7} FRT2A	5% (1/21)	72% (13/18)	5% (1/22)	0% (0/24)	5% (1/23)
Ctrl FRT2A	0% (0/25)	83% (15/18)	63% (14/22)	55% (12/22)	50% (10/22)
Percentage of testis with cyst cell clones					
ftz-f1^{ex7} FRT2A	5% (1/21)	83% (15/18)	32% (7/22)	5% (1/24)	0% (0/23)
Ctrl FRT2A	12% (3/25)	94% (17/18)	86% (19/22)	68% (15/22)	70% (14/20)

Table 4.2 *E75* clonal analysis indicates that *E75* is cell autonomously required for GSC and CySC maintenance

Genotype	0d ACI	2d ACI	8d ACI
Percentage of testis with CySC clones			
E75$^{\Delta 51}$ FRT80B	10% (2/20)	30% (7/23)	11% (4/35)
Ctrl FRT80B	9% (2/22)	36% (9/25)	25% (7/29)

Table S4.1. *ecd* is required cell autonomously in the GSCs and CySCs for their maintenance. (These data are presented graphically in Figure S4.3D.)

Genotype	0d ACI	2d ACI	4d ACI	6d ACI	8d ACI	10d ACI
Percentage of testes with GSC clones						
ecd^{2} FRT2A	12% (3/25)	64% (16/25)	31% (9/29)	0% (0/23)	2.4% (1/42)	0% (0/6)
Ctrl FRT2A	12% (2/17)	82% (28/34)	56% (19/34)	47% (7/15)	70% (20/29)	59% (14/24)
Percentage of testes with CySC clones						
ecd^{2} FRT2A	12% (3/25)	72% (18/25)	17% (5/29)	8.7% (2/23)	12% (5/42)	17% (1/6)
Ctrl FRT2A	0% (0/17)	77% (26/34)	47% (16/34)	40% (6/15)	35% (10/29)	42% (10/24)

Figure 4.1

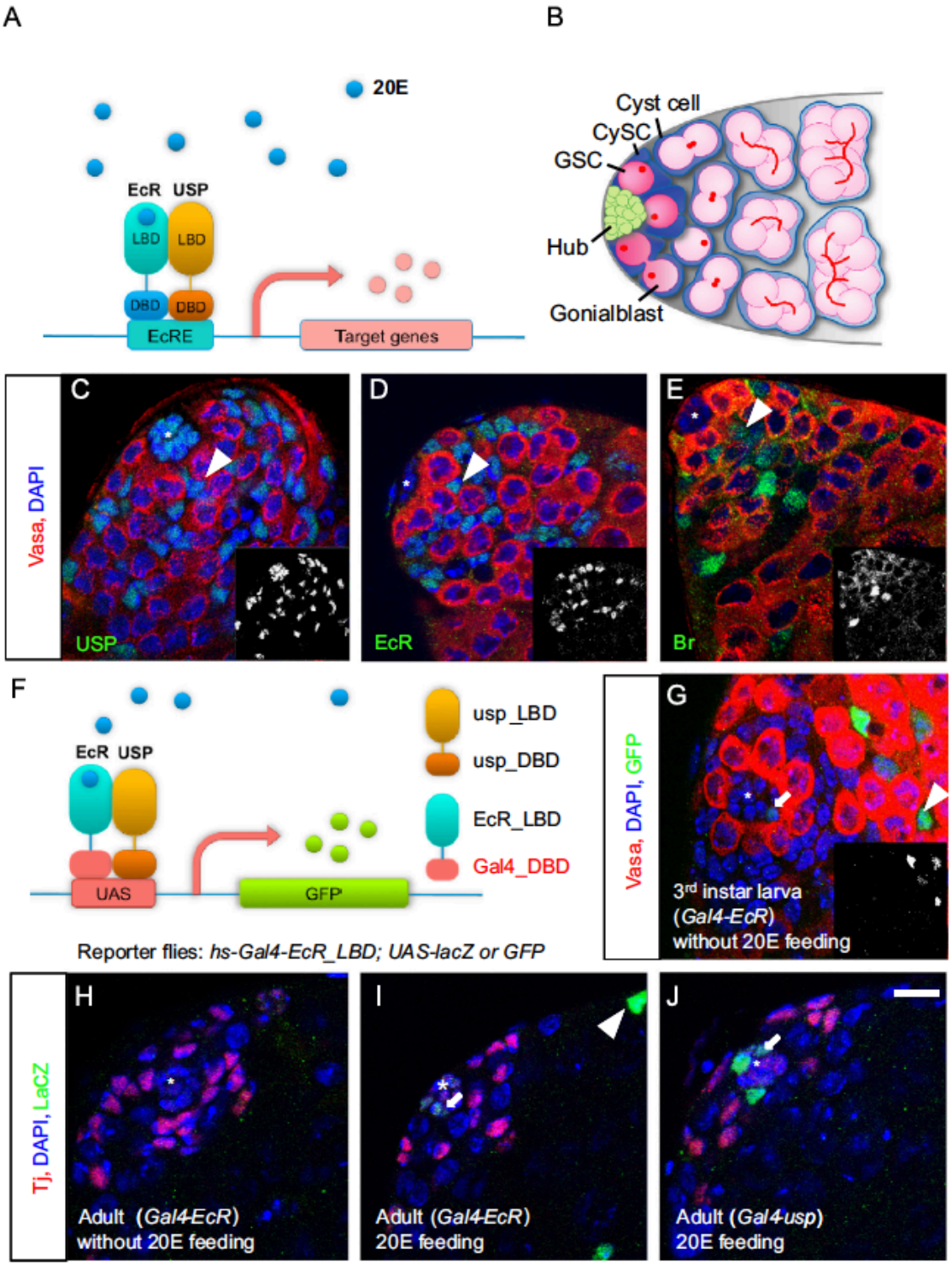
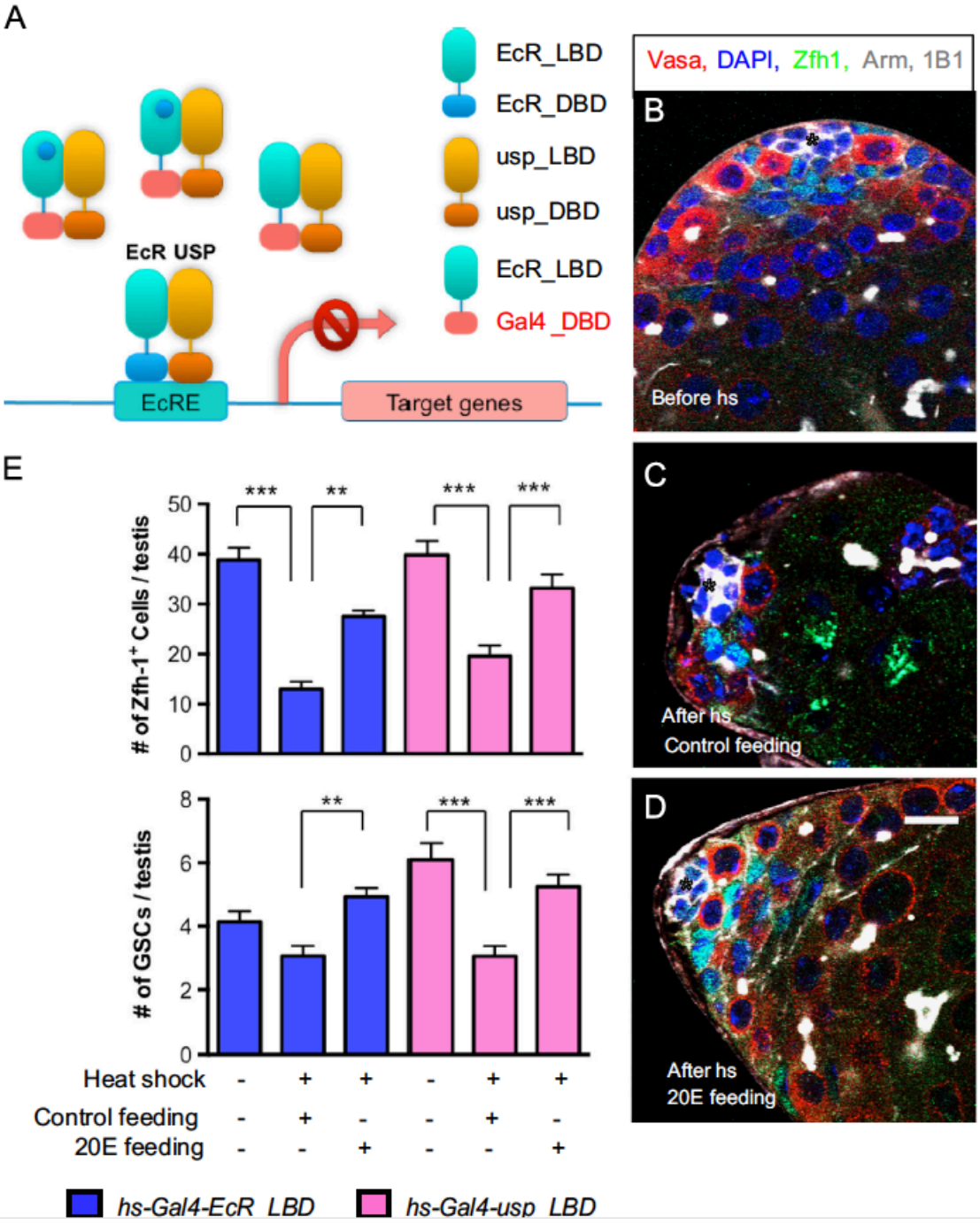

A
20E
EcR USP
LBD LBD
DBD DBD
EcRE
Target genes
B
Cyst cell
CySC
GSC
Hub
Gonialblast
C
Vasa, DAPI
USP
D
EcR
E
Br
F
EcR USP
UAS
GFP
usp_LBD
usp_DBD
EcR_LBD
Gal4_DBD
Reporter flies: hs-Gal4-EcR_LBD; UAS-lacZ or GFP
G
Vasa, DAPI, GFP
3rd instar larva
(Gal4-EcR)
without 20E feeding
H
Tj, DAPI, LaCZ
Adult (Gal4-EcR)
without 20E feeding
I
Adult (Gal4-EcR)
20E feeding
J
Adult (Gal4-usp)
20E feeding

Figure 4.2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EcR_LBD
EcR_DBD
usp_LBD
usp_DBD
EcR_LBD
Gal4 _DBD
EcR USP
EcRE
Target genes
Vasa, DAPI, Zfh1, Arm, 1B1
B
Before hs
C
After hs
Control feeding
D
After hs
20E feeding
E
# of Zfh-1+ Cells / testis
# of GSCs / testis
Heat shock
Control feeding
20E feeding
hs-Gal4-EcR LBD
hs-Gal4-usp LBD

Figure 4.3

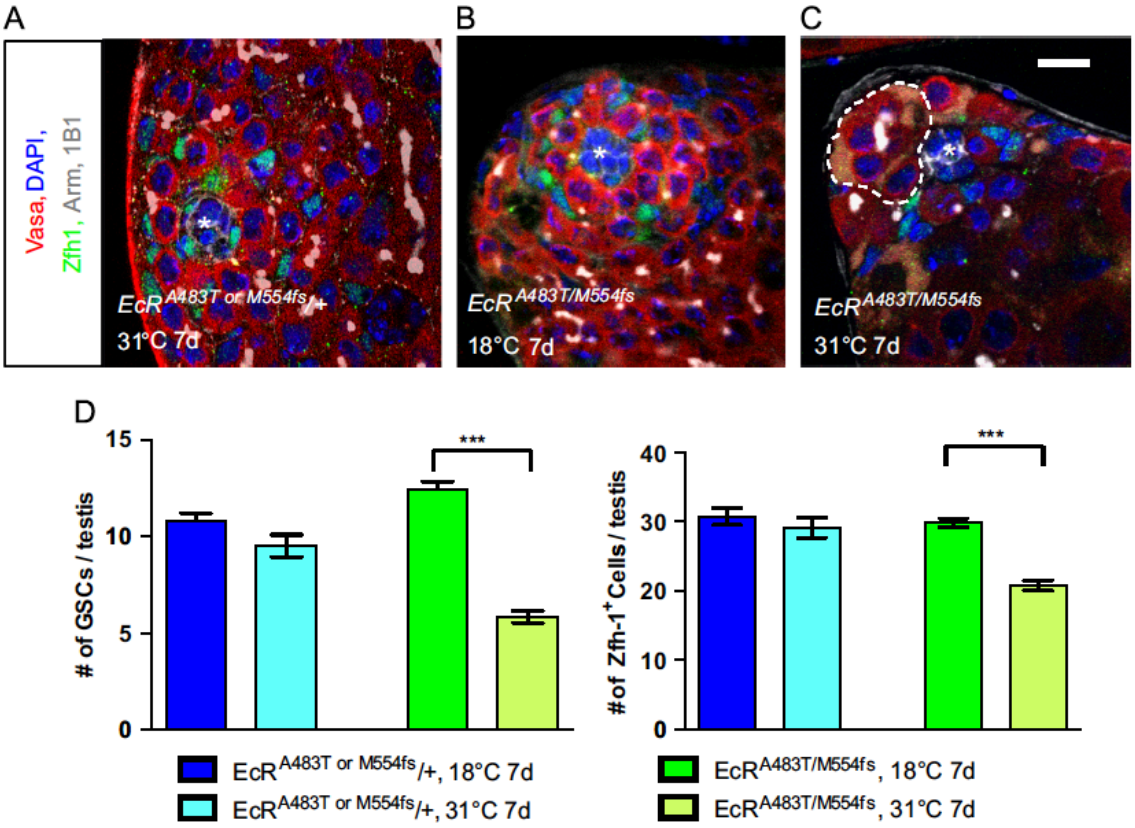
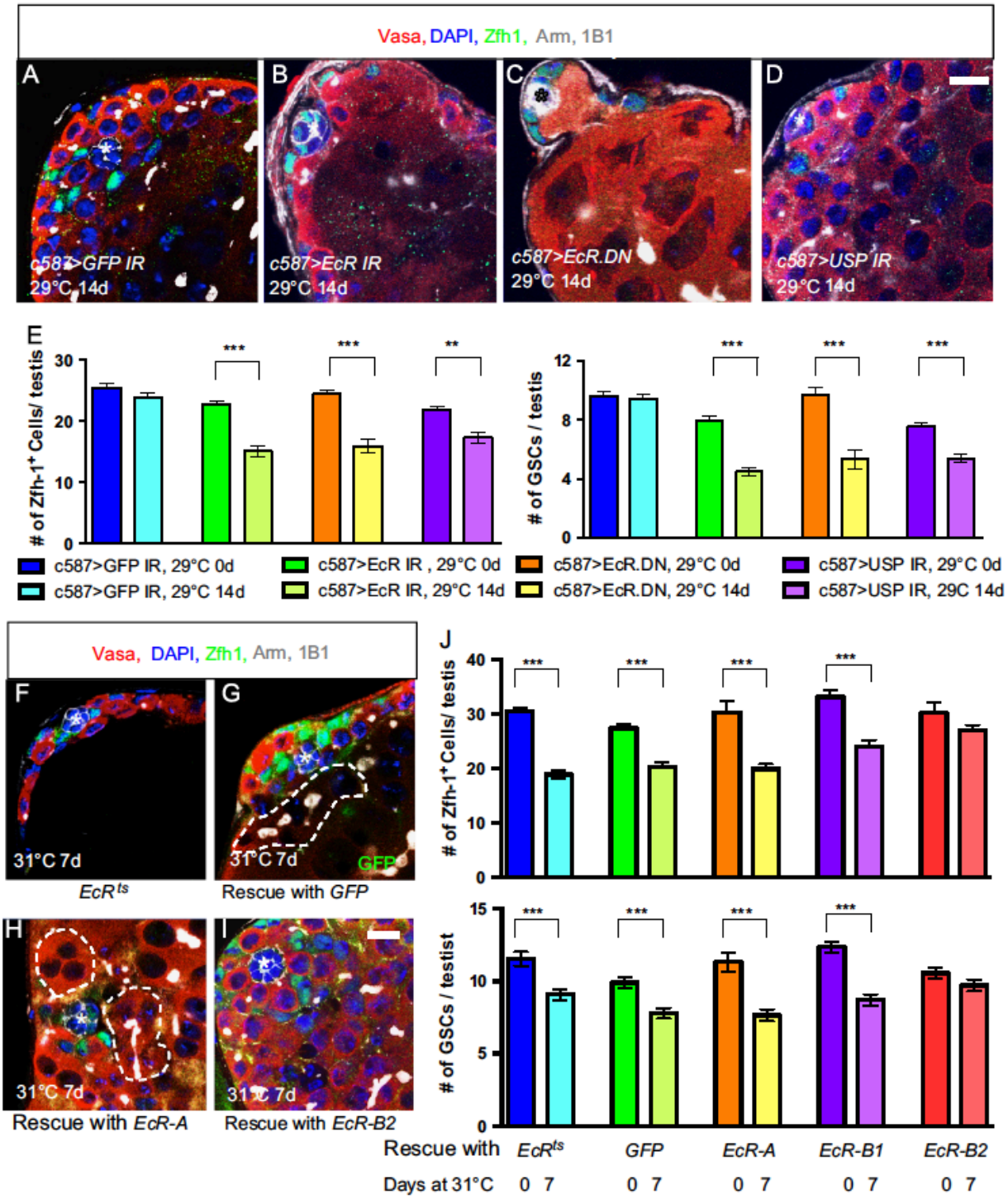

A
B
C
Vasa, DAPI,
Zfh1, Arm, 1B1
EcR A483T or M554fs /+
31°C 7d
EcR A483T/M554fs
18°C 7d
EcR A483T/M554fs
31°C 7d
D
# of GSCs / testis
15
10
5
0
***
#of Zfh-1+ Cells / testis
40
30
20
10
0
***
EcR A483T or M554fs /+, 18°C 7d
EcR A483T or M554fs /+, 31°C 7d
EcR A483T/M554fs, 18°C 7d
EcR A483T/M554fs, 31°C 7d

Figure 4.4


Vasa, DAPI, Zfh1, Arm, 1B1
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29°C 14d
B
c587>EcR IR
29°C 14d
C
c587>EcR.DN
29°C 14d
D
c587>USP IR
29°C 14d
E
# of Zfh-1+ Cells/ testis
# of GSCs / testis
c587>GFP IR, 29°C 0d
c587>GFP IR, 29°C 14d
c587>EcR IR , 29°C 0d
c587>EcR IR, 29°C 14d
c587>EcR.DN, 29°C 0d
c587>EcR.DN, 29°C 14d
c587>USP IR, 29°C 0d
c587>USP IR, 29C 14d
Vasa, DAPI, Zfh1, Arm, 1B1
F
31°C 7d
EcRts
G
31°C 7d
GFP
Rescue with GFP
H
31°C 7d
Rescue with EcR-A
I
31°C 7d
Rescue with EcR-B2
J
# of Zfh-1+ Cells/ testis
# of GSCs / testist
Rescue with EcRts GFP EcR-A EcR-B1 EcR-B2
Days at 31°C 0 7 0 7 0 7 0 7 0 7

Figure 4.5

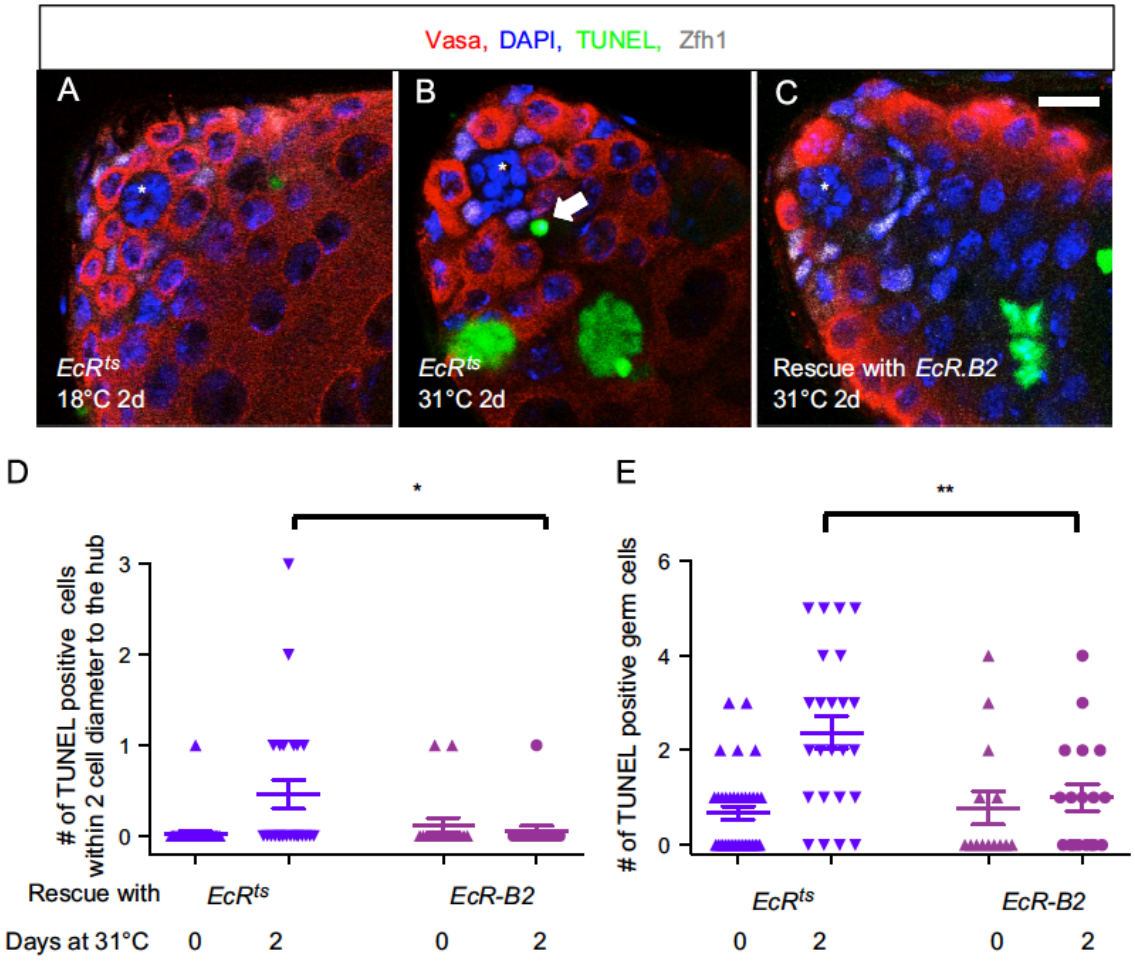
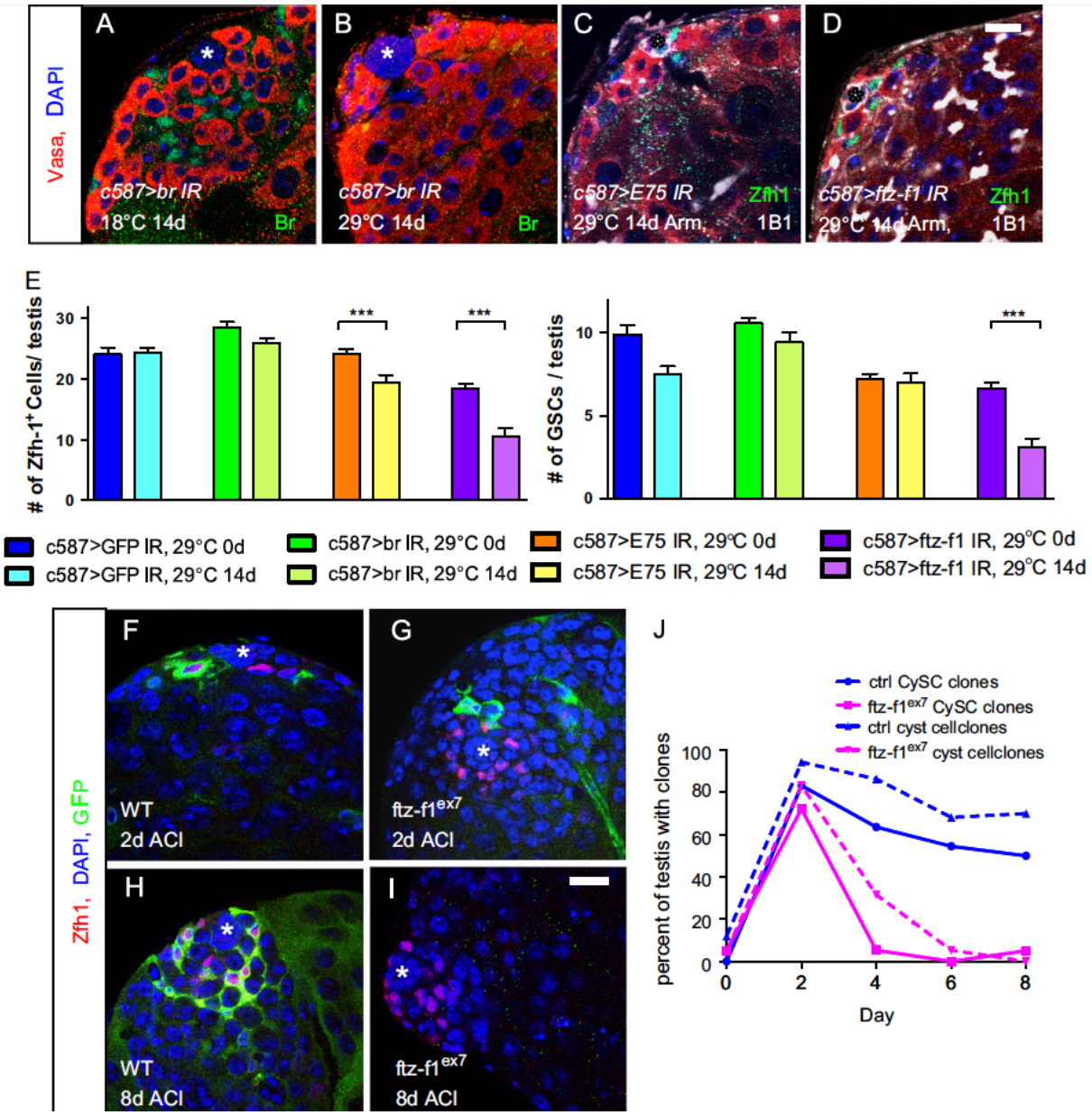

Vasa, DAPI, TUNEL, Zfh1
A
B
C
EcR^ts
18°C 2d
EcR^ts
31°C 2d
Rescue with EcR.B2
31°C 2d
D
# of TUNEL positive cells within 2 cell diameter to the hub
*
Rescue with
EcR^ts
EcR-B2
Days at 31°C
0
2
0
2
E
# of TUNEL positive germ cells
**
EcR^ts
EcR-B2
0
2
0
2

Figure 4.6


A
B
C
D
Vasa, DAPI
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18°C 14d
Br
c587>br IR
29°C 14d
Br
c587>E75 IR
29°C 14d Arm,
Zfh1
1B1
c587>ftz-f1 IR
29°C 14d Arm,
Zfh1
1B1
E
# of Zfh-1+ Cells/ testis
# of GSCs / testis
***
c587>GFP IR, 29°C 0d
c587>GFP IR, 29°C 14d
c587>br IR, 29°C 0d
c587>br IR, 29°C 14d
c587>E75 IR, 29°C 0d
c587>E75 IR, 29°C 14d
c587>ftz-f1 IR, 29°C 0d
c587>ftz-f1 IR, 29°C 14d
F
G
H
I
J
Zfh1, DAPI, GFP
WT
2d ACI
ftz-f1ex7
2d ACI
WT
8d ACI
ftz-f1ex7
8d ACI
ctrl CySC clones
ftz-f1ex7 CySC clones
ctrl cyst cellclones
ftz-f1ex7 cyst cellclones
percent of testis with clones
Day

Figure 4.7

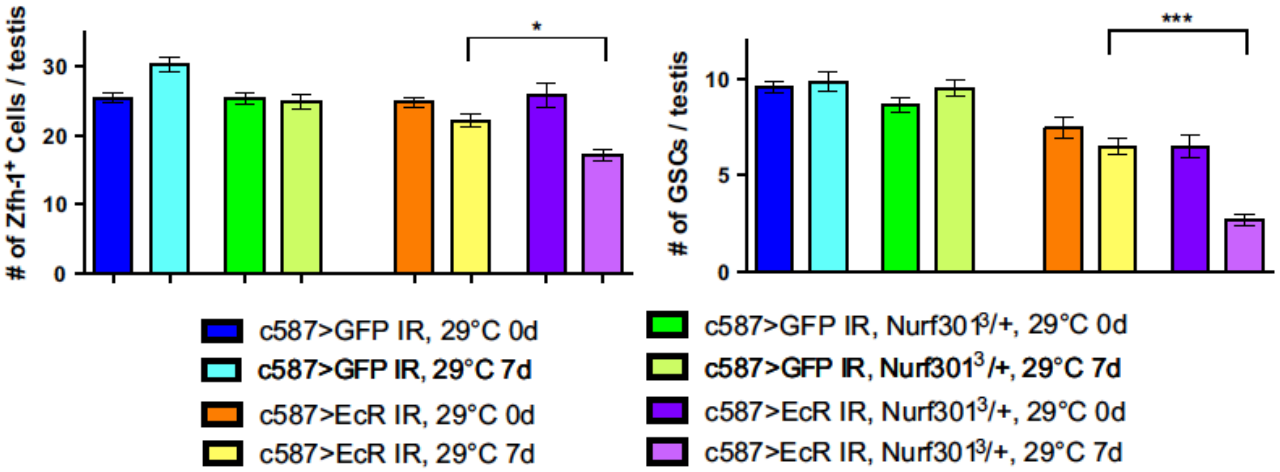

# of Zfh-1+ Cells / testis
30
20
10
0
*
# of GSCs / testis
10
5
0
***
c587>GFP IR, 29°C 0d
c587>GFP IR, 29°C 7d
c587>EcR IR, 29°C 0d
c587>EcR IR, 29°C 7d
c587>GFP IR, Nurf301^3/+, 29°C 0d
c587>GFP IR, Nurf301^3/+, 29°C 7d
c587>EcR IR, Nurf301^3/+, 29°C 0d
c587>EcR IR, Nurf301^3/+, 29°C 7d

Figure S4.1

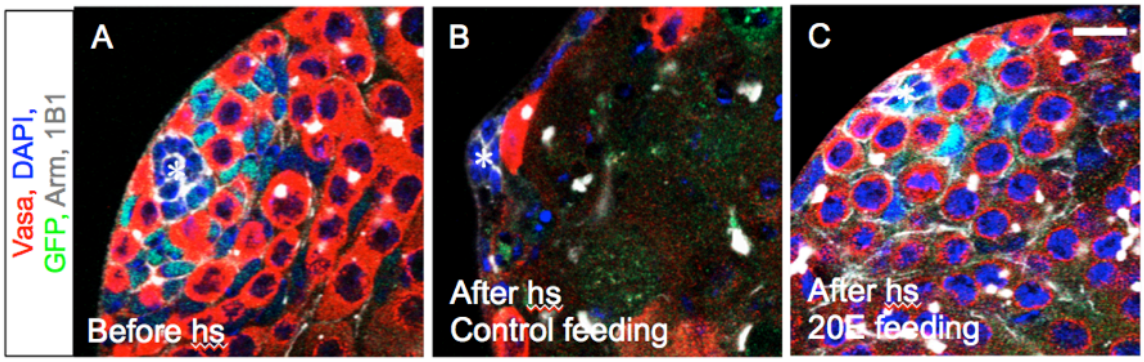

Vasa, DAPI,
GFP, Arm, 1B1
A
Before hs
B
After hs
Control feeding
C
After hs
20E feeding

Figure S4.2

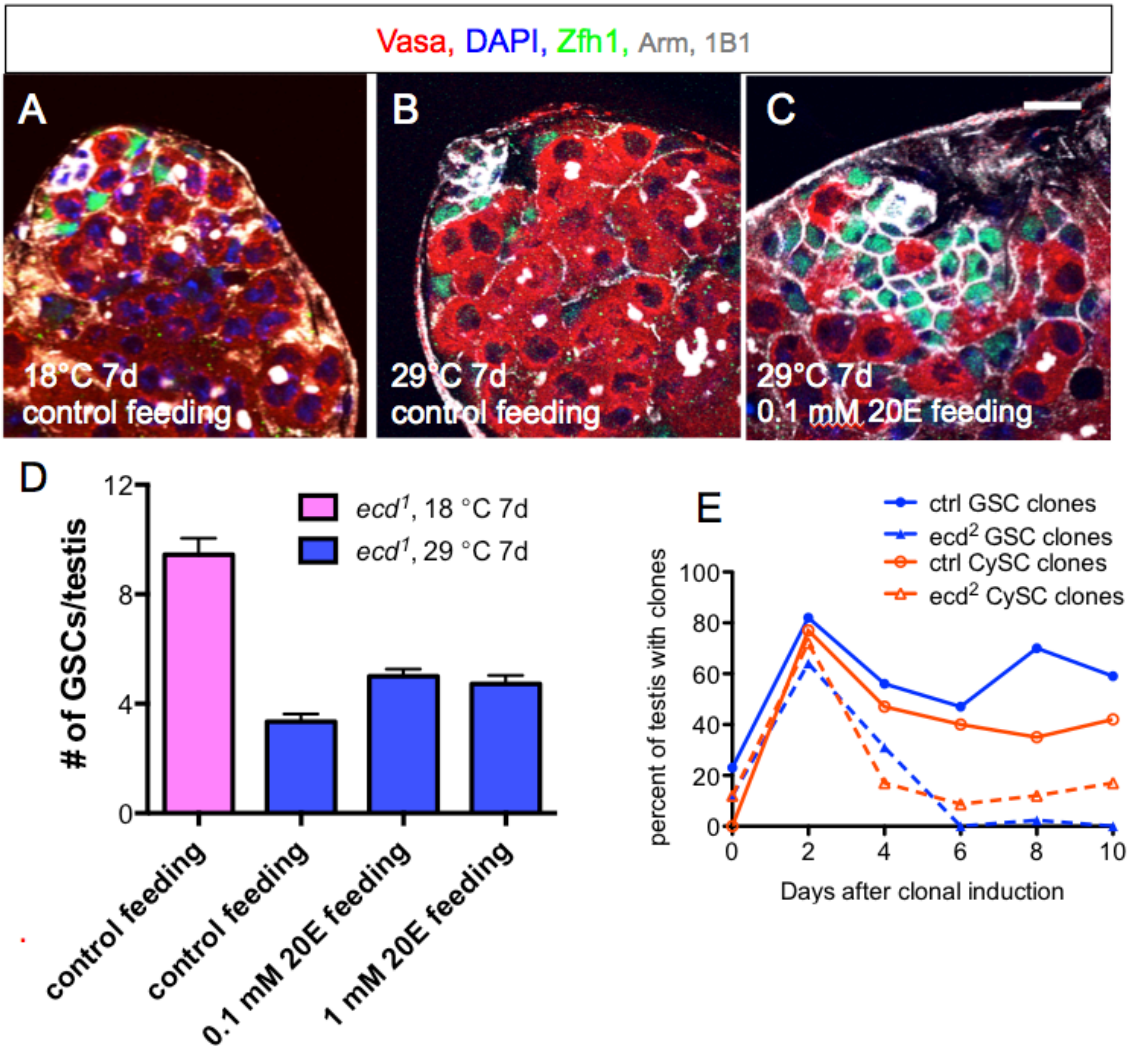

Vasa, DAPI, Zfh1, Arm, 1B1
A
18°C 7d
control feeding
B
29°C 7d
control feeding
C
29°C 7d
0.1 mM 20E feeding
D
# of GSCs/testis
12
8
4
0
ecd1, 18 °C 7d
ecd1, 29 °C 7d
control feeding
control feeding
0.1 mM 20E feeding
1 mM 20E feeding
E
percent of testis with clones
100
80
60
40
20
0
0
2
4
6
8
10
Days after clonal induction
ctrl GSC clones
ecd2 GSC clones
ctrl CySC clones
ecd2 CySC clones

Figure S2.3

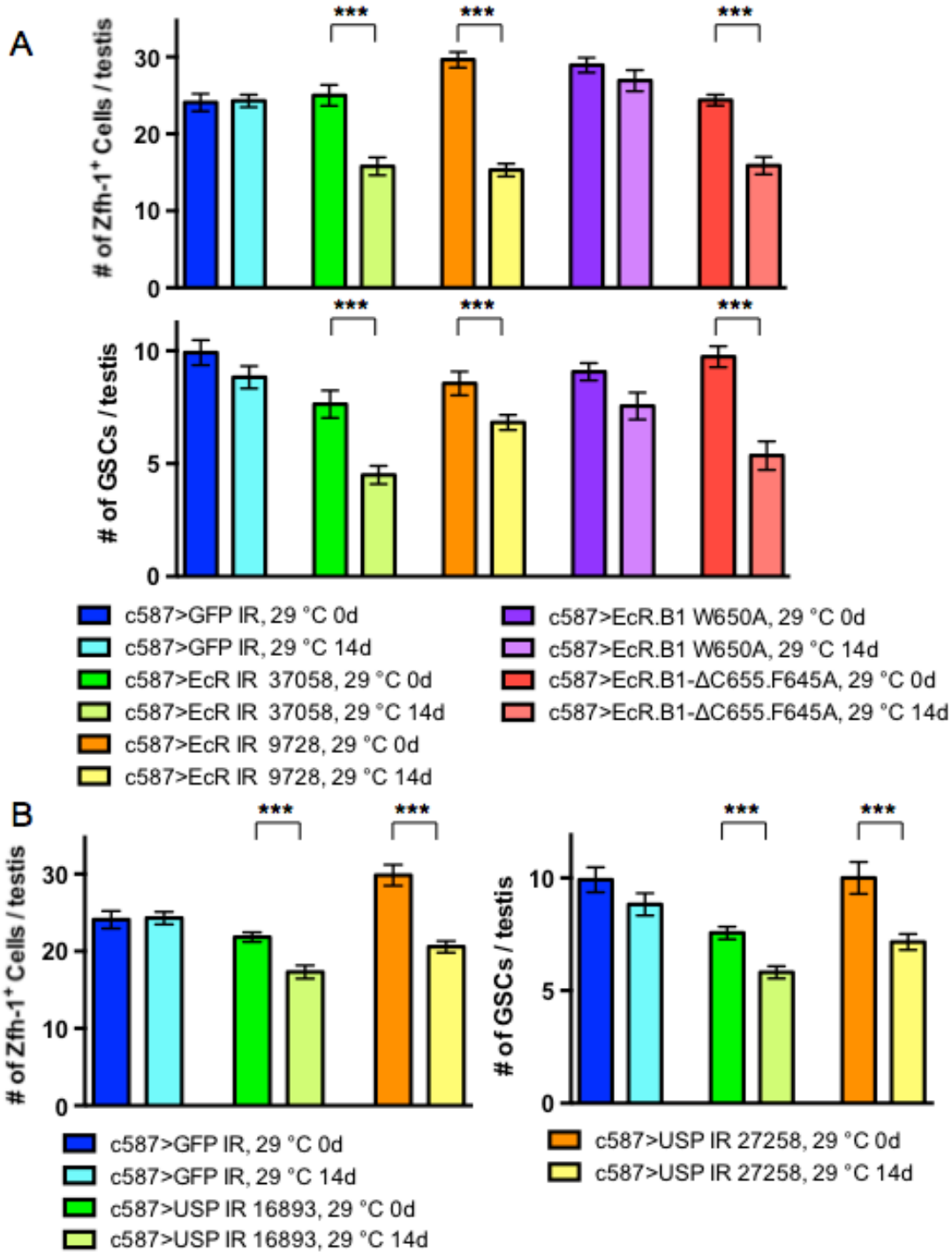
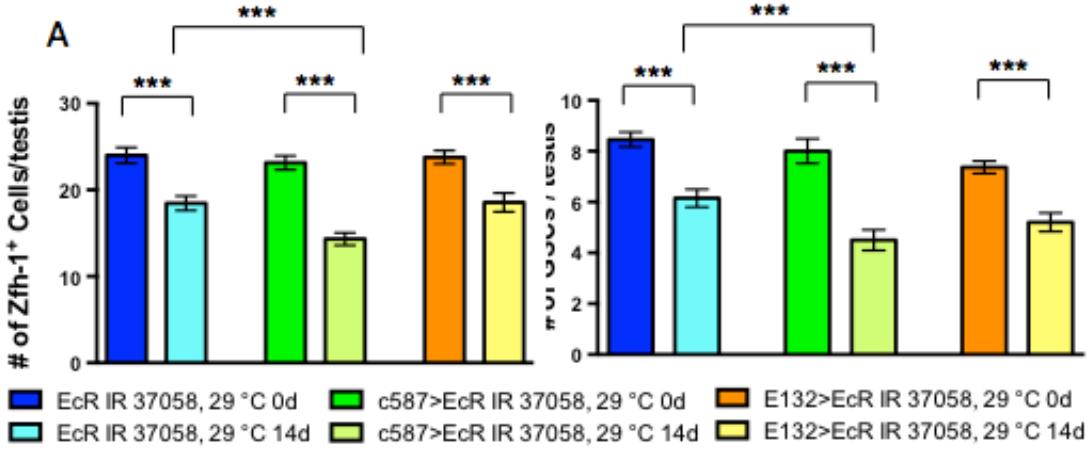

A
# of Zfh-1+ Cells / testis
# of GSCs / testis
***
c587>GFP IR, 29 °C 0d
c587>GFP IR, 29 °C 14d
c587>EcR IR 37058, 29 °C 0d
c587>EcR IR 37058, 29 °C 14d
c587>EcR IR 9728, 29 °C 0d
c587>EcR IR 9728, 29 °C 14d
c587>EcR.B1 W650A, 29 °C 0d
c587>EcR.B1 W650A, 29 °C 14d
c587>EcR.B1-ΔC655.F645A, 29 °C 0d
c587>EcR.B1-ΔC655.F645A, 29 °C 14d
B
# of Zfh-1+ Cells / testis
# of GSCs / testis
c587>GFP IR, 29 °C 0d
c587>GFP IR, 29 °C 14d
c587>USP IR 16893, 29 °C 0d
c587>USP IR 16893, 29 °C 14d
c587>USP IR 27258, 29 °C 0d
c587>USP IR 27258, 29 °C 14d

Figure S4.4


A
# of Zfh-1+ Cells/testis
EcR IR 37058, 29 °C 0d
EcR IR 37058, 29 °C 14d
c587>EcR IR 37058, 29 °C 0d
c587>EcR IR 37058, 29 °C 14d
E132>EcR IR 37058, 29 °C 0d
E132>EcR IR 37058, 29 °C 14d

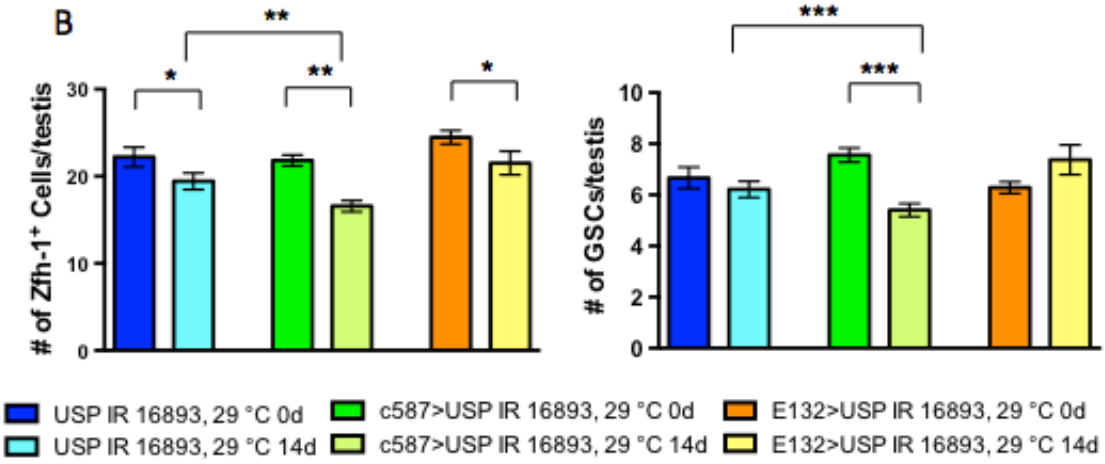
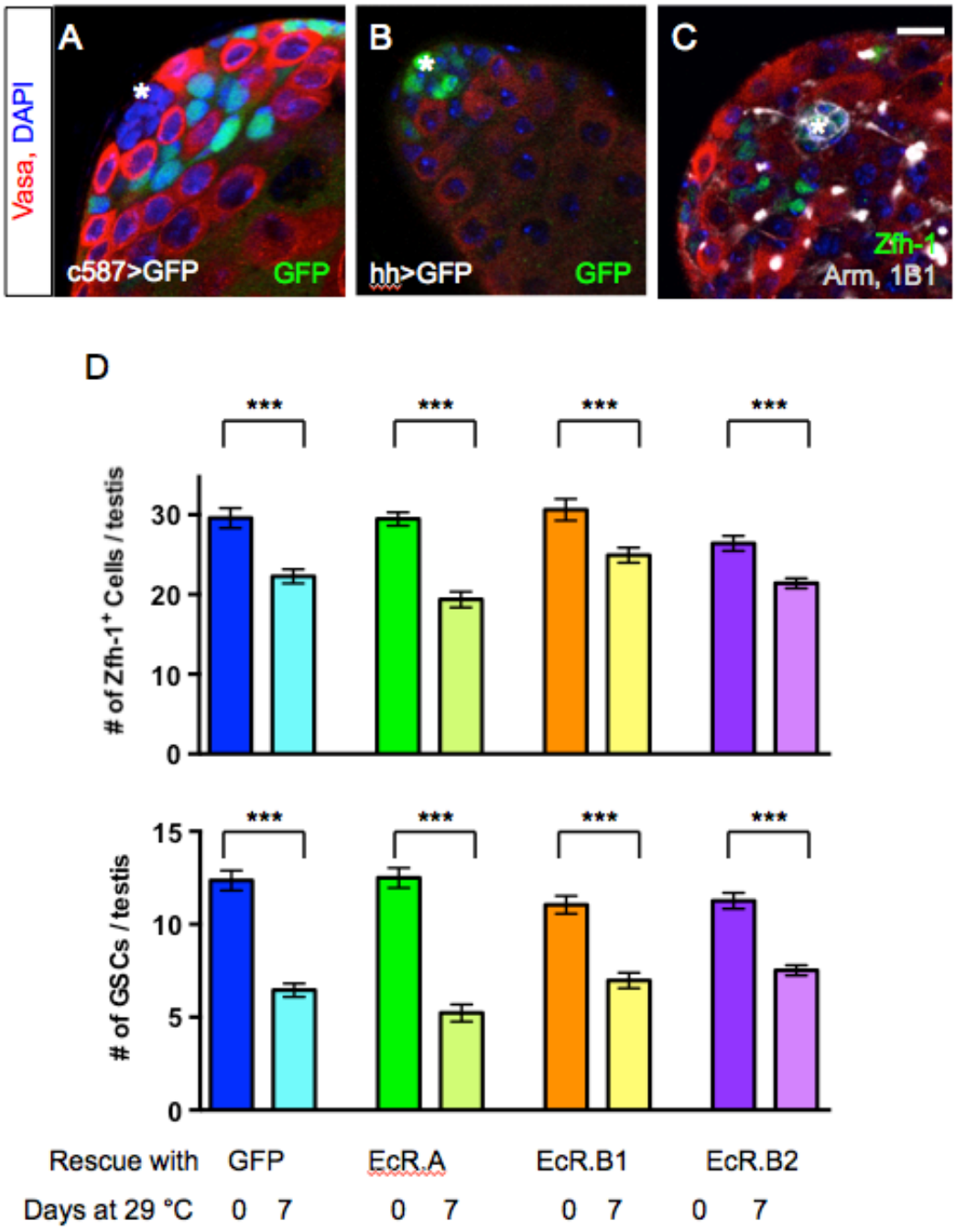

B
# of Zfh-1+ Cells/testis
# of GSCs/testis
USP IR 16893, 29 °C 0d
USP IR 16893, 29 °C 14d
c587>USP IR 16893, 29 °C 0d
c587>USP IR 16893, 29 °C 14d
E132>USP IR 16893, 29 °C 0d
E132>USP IR 16893, 29 °C 14d

Figure 4.5


Vasa, DAPI
A
c587>GFP
GFP
B
hh>GFP
GFP
C
Zfh-1
Arm, 1B1
D
# of Zfh-1+ Cells / testis
30
20
10
0
***
# of GSCs / testis
15
10
5
0
Rescue with GFP EcR.A EcR.B1 EcR.B2
Days at 29 °C 0 7 0 7 0 7 0 7

QING MA
725 N Wolfe St, Hunterian G1, Baltimore, MD, 21205
qma3@jhmi.edu

Education

2015	Ph.D.	Biochemistry, Cellular and Molecular Biology	Johns Hopkins University School of Medicine
2009	B.S.	Biological Science	Peking University, China
2009	B.S.	Economic Science	Peking University, China

Honors and Awards

2015	Outstanding Young Investigators' Award, Johns Hopkins University School of Medicine
2014	Graduate Student Association Travel Award, Johns Hopkins University School of Medicine
2014	Lewis Travel Award, Johns Hopkins University School of Medicine, Department of Cell Biology
2008	First Prize in "*Jiang Zehan"* Mathematical Contest in Modeling, Peking University. (I was the only student from College of Life Science among the first prize winners.)
2008	"Wu-Si" Fellowship, Peking University (awarded to the top student in each department)
2007	POSCO Fellowship, Peking University (awarded to the top 20 students at the University)
2004	Silver Medal, China Biology Olympiad (I got early admission to Peking University by this award)

Research Experience

2010-Present **Graduate student**, Laboratory of Erika Matunis, Department of Cell Biology, Johns Hopkins University School of Medicine

My graduate research has focused on understanding the local and systemic signals that regulate stem cell fate in the Drosophila testis stem cell niche.
I have worked on three projects:
- Function of the Jak-STAT target Chinmo in adult stem cell sex maintenance (published)
- Role of Chinmo in ovarian sex maintenance and regulation of Chinmo by microRNA let-7 (in preparation)
- Regulation of stem cell maintenance by steroid hormones (published)

2010 **Rotation student**, Laboratory of Jeremy Nathans, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine
- Studied blood vessel development in the mouse by endothelium-specific sparse labeling and clonal analysis.

- Investigated mouse lung structure in *endothelin 2* knockout mice by electron microscopy and confocal imaging.

2008-2009 **Undergraduate research fellow**, Laboratory of He-ping Cheng in the Lab of Calcium Signaling, Institute of Molecular Medicine, Peking University
- Studied in-vivo mitochondrial superoxide flashes in mouse skeletal muscle and cardiomyocytes using confocal imaging and a novel superoxide indicator.

2007-2008 **Undergraduate research fellow**, Laboratory of Qi Ouyang, Department of Physics, Peking University
- Used synthetic biology to construct a genetic circuit in yeast, which used Activation-Induced DNA-Cytosine Deaminase (AID) to induce mutations and direct the evolution of proteins.

Publications

Ma Q, Wawersik M, Matunis E. (2014) The Jak-STAT target Chinmo prevents sex transformation of adult stem cells in the Drosophila testis niche. ***Developmental Cell*** 31: 474-486**.** (**Cover article**, highlighted by ***Nature Review Genetics***, ***Developmental Cell*** and ***Biology of Reproduction***.)
http://www.nature.com/nrg/journal/vaop/ncurrent/full/nrg3872.html
http://www.cell.com/developmental-cell/abstract/S1534-5807%2814%2900694-7
http://www.biolreprod.org/content/early/2014/11/17/biolreprod.114.126862.full.pdf+html

Li Y, **Ma Q,** Cherry CM, Matunis E. (2014) Steroid signaling promotes stem cell maintenance in the Drosophila Testis. ***Developmental Biology*** 394: 129-141.

Manuscripts in Preparation

Ma Q, de Cuevas M, Matunis E. The role of Chinmo and microRNA let-7 in sex maintenance in the adult Drosophila testis and ovary niches.

Selected Platform Talks

2014 Adult somatic stem cell sex maintenance in the Drosophila testis niche
Germ Cells Meeting, Cold Spring Harbor Laboratories, Long Island, NY

2013 Chinmo prevents male-to-female sex transformation of somatic stem cells in the adult Drosophila testis
54th Annual Drosophila Research Conference, Washington DC

2008 A Circuit for Directed Evolution *in vivo*
iGEM (International Genetically Engineered Machine competition) Jamboree, MIT, Cambridge, MA

Selected Poster Presentations

2014 The Jak-STAT target Chinmo prevents sex transformation of adult stem cells in the Drosophila testis niche
Stem Cell Science and Engineering meeting, Baltimore, MD

2014 The Jak-STAT target Chinmo prevents sex transformation of adult stem cells in the Drosophila testis niche
Keystone Symposia on Stem Cells and Reprogramming, 2014, Olympic Valley, CA

2013 Ecdysone Regulation of Stem Cell Maintenance in the Drosophila Testis Niche
54th Annual Drosophila Research Conference, Washington DC

2012 Ecdysone Regulation of Stem Cell Maintenance in the Drosophila Testis Niche
53rd Annual Drosophila Research Conference, Chicago, IL

Teaching, Work and Leadership Experience

2012-2013 **Tutor,** Genetics, Graduate student course at Johns Hopkins University School of Medicine

2012 **Judge**, Roland Park Middle School and Randallstown High School science fairs

2008 **Intern,** Corning Incorporated, Science and Technology Division, Beijing, China

2008 **Team Leader and Chief Financial Manager,** Peking iGEM (International Genetically Engineered Machine competition) team

2006 **Chief of External Affairs**, Students Union, Environmental Science Department, Peking University